REMARKS

I. Specification Amendments

The specification has been amended herein to correct a variety of inadvertant errors discovered after filing. In particular, the specification has been amended in the sub-section heading at p. 16, line 29 to delete the term "1,2-alpha mannosidase" and replace it with "1,6-mannosyltransferase". Support for this amendment is found in the section of the specification following that error (pages 187-20), in which the process of finding genes encoding 1,6-mannosyltransferases to eliminate their gene products is described (see, e.g., p. 17, lines 16-27 and surrounding text; and p. 19, lines 23-24). The instant application teaches to reduce or eliminate enzymes which support hypermannosylation, such as 1,6-mannosyltransferases, and to add human-like glycosylation enzymes, such as 1,2-alpha mannosidase. Thus, the error being corrected is an obvious one.

The specification has also been amended at p. 26, line 1 to correct an inadvertent typographical error. Specifically, "sym" has been replaced by "syn."

The specification has further been amended at p. 33, line 7 to correct an inadvertent error in the title of Example 1. Specifically, "insulin" has been replaced by "interferon". Support for this amendment is found in Example 1 which follows the error. There, expression of glycosylated "interferon-beta" is described.

Similarly, at p. 33, line 10, "interferon-α" has been replaced with "interferon-β" to correct another obvious typographical error. Support for this amendment is on p. 33, lines 15, 18, 21, 23 and 24.

Finally, to comply with MPEP § 608.01, applicant has deleted two embedded hyperlinks at page18 and two at page 39 of the specification. In particular, the term "http://genome-www.stanford.edu/Saccharomyces/" (page 18, lines 1-2) has been deleted and replaced with ", e.g., see the Saccharomyces genome link at the Stanford University website"; and the terms "http://www.resgen.com/products/YEASTD.php3" (page 18, line 3); ": http://www.ebi.ac.uk/ (page 39, line 3); and ": http://www.expasy.ch/spr" (page 39, line 4) have been deleted.

II. Claim Amendments

Claims 35-64 are currently pending. With this response, claims 36-38, 41, 51, 55 and 56 have been canceled; and claims 35, 39, 40, 42-47, 49-50, 52, 54, 57-59 and 61 have been amended to define more clearly what applicant considers to be his invention. Claims 65-73 have been added and are addressed below with the discussion of claim amendments. In addition, several claims have been amended to include subject matter that was inadvertantly omitted in earlier claims.

Claim 44 has been amended to add that the glycoprotein can comprise the sugar can be N-acetylglucosamine (GlcNAc). Support for this amendment is found, e.g., at page 27, line 8. Claim 57 has been amended to broaden the recited pH range of the mannosidase enzyme to pH 5.1 to 8.0. Support for this amendment is found, e.g., at page 25, lines 11-12. Claim 58 has been amended to recite that the mannosidase domain may be from *Xanthomonas manihotas* as is disclosed in Table 3 on page 22. Each of these amendments

is supported in the application as originally filed and none adds new matter. The other claim amendments are discussed below with respect to the Examiner's rejections.

A. 35 U.S.C. § 112 Claim Rejections – Indefiniteness

Claims 35-64 stand rejected under 35 U.S.C. § 112, second paragraph, for being indefinite with repect to a variety of terms:

1. "human-like glycoprotein": According to the Examiner, the specification does not define this term clearly enough to render definite the metes and bounds of the claims. Accordingly, applicant has amended claim 35, to delete this term and replace it with "humanized glycoprotein", a term that is used interchangeably with the term "human-like glycoprotein" in the original specification. "Humanized glycoprotein" is explicitly defined in the specification as "a protein having attached thereto N-glycans including less than four mannose residues, and the synthetic intermediates (which are also useful and can be manipulated further in vitro) having at least five mannose residues" (page 13, line 25 – page 14, line 1).

2. "at least 30% $Man_5GlcNAc_2$ is produced": According to the Examiner, it is not clear what the percentage refers to. Applicant believes that such a % term is understood by the skilled worker to refer to the % molar ratio of a particular species relative to total N-glycans (see, e.g., Chiba *et al.*, *J. Biol. Chem.* 273:26298-26304 (Oct. 1998)) ("Chiba"), which is discussed in more detail below). For clarity, the claims have thus been amended to recite "in excess of 30 mole % of the N-glycan structures". Original support for this amendment may be found, e.g., at page 16, lines 6 – 28 (see lines 11 and 20 for support for

"in excess of 30%"). And support for the molar percentage term referring to "% of the total N-glycans" may further be found at page 16, lines 24-25 (see also page 11, lines 5-10 and page 23, lines 12-14).

3. "representative enzymes in the organelle": The Examiner contends that this term is indefinite as there are no clear criteria for determining, in any given organelle, what a "representative enzyme" is. Applicant has thus amended this term to refer to "glycosylation-related enzymes in the subcellular location where the domain is targeted." Support for this amendment may be found, e.g., at page 21, lines 9-15 and Table 4, page 24 (the title of which includes the term "glycosylation related enzymes" and which discloses a variety of glycosylation enzymes located in the ER and Golgi and their pH optima). Accordingly, applicant requests that the Examiner withdraw this rejection.

4. "NeuNAc-Gal-GlcNAc-Man": The Examiner contends that the term "NeuNAc-Gal-GlcNAc-Man" renders claim 45 indefinite because the specification does not teach such a molecule, nor what the preface "Neu" stands for. Applicant refers the Examiner to page 2, lines 23-24 where "Neu" is defined as "neuraminic acid" and "Ac" is defined as "acetyl". "NAc" is well-known to the skilled worker as specifying an N-acetyl linkage. Accordingly, NeuNAc refers to *N*-acetyl neuraminic acid, also commonly known as "sialic acid," a hallmark of a humanized glycoprotein, which is also often abbreviated "NANA" (see Figure 1B and accompanying text at page 3, lines 10-12: "[c]ommonly the terminal residues of the *N*-glycans [of animal glycoproteins] consist of sialic acid. A typical structure of a human *N*-glycan is shown in Figure 1B"; see also page 6, line 21 – page 7, line 9 for the importance of having terminal sialic acid on a humanized glycoprotein).

"Gal" is defined on page 2, line 19 as "galactose"; "GlcNAc" is defined on page 27, line 8 as "*N*-acetylglucosamine". The term "Man", which is well-known in the art as referring to "mannose", can easily be deduced from, e.g., page 10, line 4: "...which adds a mannose to a glycan structure $Man_8GlcNAc_2$ to yield $Man_9GlcNAc_2$." Applicant believes that one of skill in the art would readily understand the meaning of the term "NeuNAc-Gal-GlcNAc-Man" (with or without reference to the disclosure and Figure 1B) and that claim 45 is thus definite. Reconsideration of this rejection is respectfully requested.

5. "derived": The Examiner contends that the term "derived" in claims 58 and 61 renders those claims indefinite. For clarity, applicant has amended those claims to delete the term "derived" thus obviating this rejection.

6. "one enzyme": The Examiner contends that it is unclear which enzyme this term refers to. Claim 59 (through claim 35, from which it depends) has been amended for clarity, thus obviating this rejection.

7. "catalytic domain": The Examiner contends there is insufficient antecedent basis for the limitation of "catalytic domain" in line 1 of claim 61. Claim 61 has been amended for clarity, thus obviating this rejection.

8. Omitted essential steps: Claims 35-64 stand rejected under 35 U.S.C. 112, second paragraph, for being incomplete for omitting essential steps. The Examiner asserts that "how to determine that a human-like glycoprotein is produced" is an essential step and thus must be recited in the claims. Applicant traverses. Because amended claim 35 recites a "humanized glycoprotein", whose explicit definition (see page 13, line 25 – page 14, line

1) encompasses the $Man_5GlcNAc_2$ intermediate recited in that claim, the claim does not omit an essential element. That is, the production of the $Man_5GlcNAc_2$ intermediate in excess of 30% of the total N-glycans constitutes production of a humanized glycoprotein. Accordingly, applicant requests that this rejection be withdrawn.

9. Conclusion: For the above reasons, applicant respectfully requests that the Examiner withdraw the pending rejections based on indefiniteness.

B. 35 U.S.C. § 112 Claim Rejections – Written Description

Claims 35-64 stand rejected under 35 U.S.C. § 112, second paragraph, for lack of adequate written support in the application as originally filed. The Examiner's specific rejections are addressed below.

1. Host cells

Pending claims 35-64 are directed to methods for producing human-like glycoproteins in a lower eukaryotic host cell. The Examiner contends that the claimed methods require use of a lower eukaryotic host cell belonging to a genus of cells (i.e., ones that do not display a 1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein) that is overly broad in view of the disclosure of two host species within that genus (*OCH1* mutant strains of *Saccharomyces cerevisiae* and *Pichia pastoris*) that lack 1,6 mannosyltransferase activity.

Applicant respectfully disagrees. The present invention is directed to methods for engineering human-like glycoproteins in *any* lower eukaryotic host cell that lacks 1,6

mannosyltransferase activity (associated with hypermannosylation of glycoproteins), produced or acquired by any means. *Pichia pastoris* and *Saccharomyces cerevisiae* are but two examples of lower eukaryotic host cells in which 1,6 mannosyltransferase activity has been depleted. These examples, however, are in no way limiting with respect to the methods of the invention. As stated in the instant application:

> In a preferred embodiment, eukaryotic strains which do not express one or more enzymes involved in the production of high mannose structures are used. These strains can be engineered or one of the many such mutants already described in yeasts, including a hypermannosylation-minus (OCH1) mutant in Pichia *pastoris.*
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> The method described herein may be used to engineer the glycosylation pattern of a wide range of lower eukaryotes (e.g. *Hansenula polymorpha, Pichia stiptis, Pichia methanolica, Pichia sp, Kluyveromyces sp, Candida albicans, Aspergillus nidulans, Trichoderma reseei etc.). Pichia pastoris* is used to exemplify the required manipulation steps.

(page 14, lines 7-10 and page 17, lines 1-5)

A person skilled in the art having read the instant application would comprehend and be able to make and/or otherwise identify a variety of other lower eukaryotic host cells lacking 1,6 mannosyltransferase activity using techniques well-known in the art. The application, for example, teaches that a host cell lacking 1,6 mannosyltransferase activity may be obtained by: **(a)** directed mutation of an *OCH1* homolog (see, e.g., p. 18, line 22 - page 20, line 6); **(b)** mutagenesis and classical genetic screens in lower eukaryotic organisms (page 17, line16 – page 18, line 21); or **(c)** identifying a host that is naturally deficient in such activity (such as filamentous fungi; see, e.g., page 17, lines 13-14 and

discussed below). And, it is well established that the description need only describe in detail that which is new or not conventional. *See Hybritech* v. *Monoclonal Antibodies*, 802 F.2d at 1384, 231 USPQ at 94; *Fonar Corp.* v. *General Electric Co.*, 107 F.3d at 1549, 41 USPQ2d at 1805. According to the MPEP, "[t]his is equally true whether the claimed invention is directed to a product or a process." (MPEP § 2163, section II.A.3(a)). At the original filing date, it was within the skill of the art to isolate or make, test and identify lower eukaryotic host cells deficient in 1,6 mannosyltransferase activity using standard biological techniques.

The Examiner, focusing on disclosed method **(a)** (directed mutation of an *OCH1* homolog) for producing the claimed host cell, contends that there is no teaching that the *OCH1* gene encodes a 1,6 mannosyltransferase activity in other species of lower eukaryotic host cells encompassed by the claims, and thus that the specification teaches merely functional but not structural characteristics of the cell required for use in the claimed methods (Office Action, pp. 3-4). As explained above, however, the invention is not limited to methods for producing glycoproteins in a mutant *OCH1* host cell, although such mutant host cells are one preferred host cell that lacks alpha-1,6 mannosyltransferase activity.

Moreover, applicant disagrees with the Examiner's characterization of the claim as lacking structural features. The term "lower eukaryotic host cell" provides the "structural" characteristics that the Examiner contends are lacking in claim 35. The term "that does not display alpha-1,6 mannosyltransferase activity with respect to the N-glycan of a glycoprotein" functionally modifies that art-defined structure. Disclosed functional

characteristics, when coupled with a known or disclosed correlation between function and structure, suffice for written description (MPEP § 2163, section II.A.3(a)).

Disclosed method **(b)** (mutagenesis and classical genetic screens in lower eukaryotic organisms) could also be used to produce lower eukaryotic host cells that lack alpha-1,6 mannosyltransferase activity. Loss of this enzymatic activity are determined by methods well known in the art, e.g., high performance liquid chromotagraphy (HPLC) and/or high resolution ^{1}H NMR (Nakanishi-Shindo et al., 1993; *J. Biol. Chem.*, 268: 26338-25345). Assays for this a-1,6 mannosyltransferase activity were also well-known in the art (see, e.g, Nakayama et al., 1997, *FEBS Lett.*, 412: 547-550).

Finally, as to method **(c)** above, there were lower eukaryotic host cells that lacked alpha-1,6 mannosyltransferase activity which were known at the earliest effective filing date of the instant application. It was known to the skilled worker at the priority date that *Trichoderma* and *Aspergillus* species lack α-1,6 mannosyltransferase activity. *See, e.g.*, Maras et al. *Glycoconjugate J.*, 16:99-107 (1999) (attached as **Exhibit A**). Figure 1 of this reference shows glycan structures lacking a-1,6 mannose on glycoproteins from *Aspergillus niger, A. saitoi* and *A. oryzae*. Figure 2 of this same reference shows the glycan structure lacking α-1,6 mannose on a glycoprotein expressed in *Trichoderma reesei*. Thus, at the time of filing, one skilled in the art could determine that these Aspergillus and Trichoderma species lack α-1,6 mannosyltransferase activity.

Applicant and his co-workers, subsequent to the filing date of the instant application, have produced other species of lower eukaryotic cells that lack alpha-1,6

mannosyltransferase and that are useful in the methods of the invention to produce high levels of $Man_5GlcNAc_2$ intermediates: *Kluyveromyces lactis* (lacking α-1,6 mannosyltransferase by gene knock of *OCH1)*and *Aspergillus niger* (naturally lacking α-1,6 mannosyltransferase, as discussed above). Applicant has attached hereto as **Exhibit B** an example from U.S. application 10/371,877, a continuation-in-part that claims priority from the instant application.[1] This Example details the making of a *K. lactis* host strain useful for producing humanized glycoproteins according to the methods of the instant invention. Upon request by the Examiner, applicant can submit additional evidence in a declaration to show that lower eukaryotic host strains other than the two species disclosed may be made according to the teachings of the original application without undue experimentation.

In conclusion, the application as filed sufficiently teaches the skilled worker how to engineer or select lower eukaryotic cells that do not display alpha-1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein. Accordingly, applicant requests that the 112 rejection with repect to lower eukaryotic host cells be withdrawn.

2. Enzymes for $Man_5GlcNAc_2$ Production

The Examiner has objected to the term "one or more enzymes for the production of a $Man_5GlcNAc_2$ carbohydrate structure" as being overly broad (p. 4). While applicant respectfully disagrees that this term is too broad in view of the scope of the invention

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[1] The *K. lactis* example attached hereto as **Exhibit B** is from U.S. application 10/371,877, which published as US 2004/0018590 A1 (1/29/04) and which is a continuation-in-part of the instant application.

disclosure, to expedite prosecution, the claims have been amended to recite: "a mannosidase enzyme for the production of a $Man_5GlcNAc_2$ carbohydrate structure." Applicant reserves the right to pursue claims directed to the canceled subject matter in one or more later filed applications claiming priority from the instant application.

The Examiner further contends that the specification fails to disclose "other kinds of mannosidase that can produce $Man_5GlcNAc_2$ carbohydrate structure, or 1,2 mannosidase from other species that can produce $Man_5GlcNAc_2$ carbohydrate structure" (p. 4). As is known to one skilled in the art, only an α-1,2 mannosidase (mannosidase I) enzyme will catalyze the conversion of $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$. As for information regarding α–1,2 mannosidases from other species, applicant refers the Examiner to Table 3, page 22, for a list of mannosidase I enzymes from various species (*A. saitoi*, *T. reesei*, *P. citrinum*, *A. nidulans*, *H. sapiens*, *Lepidopteran*, *X. manihotis*, *Mouse* and *Bacillus*) and their corresponding pH optimum. The instant application teaches that, depending on the host cell, it may be desirable to introduce more than one α-1,2 mannosidase gene for the production of $Man_5GlcNAc_2$ (page 25, lines 8-14). This can readily be determined by one skilled in the art without undue experimentation.

For the above reasons, applicant respectfully requests that the Examiner withdraw the outstanding 112, paragraph 1 rejections based on lack of adequate written description.

C. 35 U.S.C. § 112 Claim Rejections – Enablement

Claims 35-64 stand rejected under 35 U.S.C. 112, first paragraph, for lack of enablement. In particular, the Examiner contends that the specification fails to teach the

skilled worker a method that can produce $Man_5GlcNAc_2$ carbohydrate structure on over 30% of the total glycoprotein produced by a lower eukaryotic host cell. The Examiner also asserts that there are no working examples for the claimed method and that the skilled worker would have to rely solely on the teaching of the prior art and would require undue experimentation to achieve the claimed method. Applicant traverses, for reasons explained in detail below.

1. The State of the Art and Level of Predictability at the Time of Filing

According to the Examiner, Chiba *et al.* (*J. Biol. Chem.* 273:26298-26304 (Oct. 1998)) ("Chiba") reports for the first time a yeast system in which a glycoprotein having a $Man_5GlcNAc_2$ carbohydrate structure is produced (at a level of 27% of the total glycoprotein), by introducing a hybrid α-1,2 mannosidase into a yeast host strain that lacks α-1,6 mannosyltransferase activity. The Examiner focuses on the teachings of Chiba and the level of unpredictability in Chiba's methods to reject the instant claims for lack of enablement. Applicant agrees with the Examiner on the shortcomings of the Chiba methods. As described below, the instant invention provides important improvements over Chiba's method so that one of skill in the art can make and identify a mannosidase enzyme that will produce more than 30% $Man_5GlcNAc_2$ in a selected lower eukaryotic host cell. And, the steps that are to be performed to "modify the method taught by Chiba" (Office Action, p. 7) are set forth in the original specification such that one of skill in the art would NOT have to engage in undue experimentation to practice the claimed method.

The problems with the approach of Chiba are discussed throughout the instant application (see, e.g., p. 10, line 18 to page 11, line 18; page 20, lines 15-page 21, line 9; page 23, lines 12-23). The application suggests that the process of Chiba may be inefficient because the *A. saitoi* mannosidase catalytic domain has a pH optimum of about 5.0, which is significantly lower than the pH optima of other representative enzymes in the ER (pH 6.5 – 7.5) where the *A. saitoi* mannosidase domain was targeted (using an HDEL targeting sequence). The instant application states that the activity of the *A. saitoi* mannosidase catalytic domain in the ER is less than 10% (p. 23, lines 21-23). Thus the instant application teachces that other mannosidase catalytic domains should be used for efficient production of $Man_5GlcNAc_2$ *in vivo* (page 21, lines 3-16).

2. The Invention Improves Prior Art Methods of Making Human-like Glycoproteins

Applicant's invention solves the problems of inefficiency in the prior art methods. The invention is based in part on the discovery that certain heterologous catalytic domains function much better than others in a particular subcellular location when attached to a given targeting domain of a lower eukaryotic host cell; and likewise, that the same heterologous mannosidase catalytic domain will behave differently when attached to different targeting domains which influence the subcellular location in the glycosylation pathway of a lower eukaryotic cell. In fact, some catalytic domains may not generate appreciable levels of $Man_5GlcNAc_2$ regardless of how they are targeted within the host cell secretory pathway (see, e.g., page 21, lines 7-9). This discovery inspired the methods and combinatorial libraries of the invention involving making and using hybrid enzymes

selected for their ability to function efficiently at the location they are targeted in a particular host cell of choice.

Applicant's novel method for producing glycoproteins results in substantially enhanced production of $Man_5GlcNAc_2$ glycosylation intermediates previously unattainable by prior art methods (such as Chiba). Specifically, the novel method involves introducing into the host cell a hybrid glycosylation enzyme that has been *selected* for the ability of the catalytic domain and targeting sequence to work together optimally at the location in the host cell to which they are targeted. Moreover, the invention provides improved hybrid mannosidase (and other glycosylation) enzymes comprising catalytic domains that function better than any prior art hybrid mannosidase (or other glycosylation enzyme) domains because they are selected for optimal activity (e.g., by having an appropriate pH optimum) at the location where they are targeted in the host cell in which they are expressed.

While the prior art proposes several solutions to overcoming the deficiency of inefficient $Man_5GlcNAc_2$ production (see, e.g., Chiba at page 26303, end of column 1 and beginning of column 2) to date (i.e., in over six years of subsequent research) none of these solutions have been shown to work. In contrast, the considerable direction and guidance provided by the present invention has allowed not only for the production of essentially pure $Man_5GlcNAc_2$ but has also allowed for the further modification to $GlcNAcMan_5GlcNAc_2$ *in vivo*, a feat not previously accomplished in a yeast.

While the prior art guided the skilled worker to increase expression of mannosidase using stronger promoters and other induction techniques, the present invention teaches that

it is the choice of mannosidase and its heterologous leader peptide that is the rate limiting factor. The instant application teaches that optimized hybrid mannosidases of the invention can be integrated into the chromosome of the host cell in single copy, and additional hybrid glycosylation enzymes may be similarly introduced, facilitating the engineering of complex human-like glycosylation pathways in lower eukaryotic cells (see, e.g., p. 30, lines 20-28). Contrary to the methods provided by the prior art, the present invention can be successfully practiced without requiring elevated expression levels.

This essential technical difference between the invention and Chiba is recited in amended claim 35, which requires the step of introducing a hybrid mannosidase enzyme comprising: (i) a catalytic domain selected to have a pH optimum within 1.4 pH units of the average pH optimum of glycosylation-related enzymes in the subcellular location where the domain is targeted; and (ii) a cellular targeting signal peptide (not normally associated with the catalytic domain) selected to target the catalytic domain to a subcellular location where the domain will exhibit optimal activity. Chiba neither discloses nor suggests such a hybrid mannosidase, as discussed below in more detail (see section D, page 33).

The application teaches a method for producing and identifying optimal combinations of subcellular targeting sequences coupled to glycosylation-related catalytic domains for a particular lower eukaryotic host cell of choice. An optimized hybrid enzyme can be produced using a DNA library of one or more targeting sequences linked to one or more catalytic domains which is introduced into the host cell of choice. Clones are screened for their ability to produce human-like glycoproteins (i.e., $Man_5GlcNAc_2$ and/or other human-like glycan intermediates such as those derived from $Man_5GlcNAc_2$) and

optimized hybrid enzymes may be isolated and identified. Such optimized enzymes are useful, e.g., in methods for producing humanized glycoproteins such as the method of claim 35 and claims which depend therefrom.

Amended claims 40 and 54 (which depend from amended claim 35) further require the step of introducing one or more enzymes (mannosidases, glycosyltransferases or glycosidases) in addition to the hybrid mannosidase enzyme recited in amended claim 35 (amended claim 54 introducing said additional enzyme activity or activities by means of expression from nucleic acid molecules).

Added claim 70 recites a method for producing humanized glycoproteins in a host cell that produces high levels (i.e., in excess of 30 mole %) of $Man_5GlcNAc_2$ structures (e.g., one produced using the method of amended claim 35 or claims that depend therefrom) and requires the step of introducing a hybrid GlcNAc Transferase I (GnTI) enzyme comprising a catalytic domain selected to have a pH optimum in a range of 1.4 pH units of representative glycosylation enzymes at the location to which the domain is targeted. Original support for this claim is found, e.g., at page 24 (Table 4), page 25, lines 15-20; page 27, lines 5-11; page 33, line 25 – page 34, line 9; page 37 (Table 6); page 39 (Table 7), lines 6-13; and original claims 10, 12 and 16.

Added claim 71 recites a method for producing humanized glycoproteins in a host cell comprising the step of introducing a hybrid GlcNAc Transferase I enzyme of the invention into the host cell. Original support for claim 71 is found, e.g., at page 27, lines 5-11; page 33, line 25 – page 34, line 9; page 38 (Table 6); page 39, lines 6-13 (Table 7); and,

e.g., original claims 10, 12 and 16. Added claim 72, which depends from claim 71, further requires that a nucleic acid encoding UDP-GlcNAc transporter be introduced into the host cell (as disclosed, e.g., at page 32, lines 11-20 and page 35, lines 15-22).

Finally, added claim 73 requires that N-glycans from glycoproteins produced by the host cells of claims 35, 40 or 54 be analyzed by a variety of recited methods as disclosed in the original specification, e.g., at page 31, line 14 - page 32, line 9; page 33, lines 22-24 and original claim 31.

3. Post-filing Date Evidence that the Claimed Invention is Enabled

Experiments performed after the filing date of the instant application support the enablement of the claimed methods. For example, Example 1 at page 33, discloses using the catalytic domain of the human mannosidase IB (α-1,2 mannosidase) fused to a sublibrary of targeting sequences (see also page 29, line 10 to page 30, line 3). And Table 5 on page 29 shows a list of suitable compartmental targeting sequences, including an *MNS1* targeting sequence. Subsequent work by applicant has demonstrated that human mannosidase B fused to the targeting sequence of *S. cerevisiae* (page 29, Table 5) results in 40-60% $Man_5GlcNAc_2$ produced *in vivo*. Furthermore, following the teachings of the instant application, the inventors have data showing that a mouse mannosidase IB catalytic domain (page 22, Table 3) fused to an Och1p targeting sequence (page 29, Table 5) results in 60-80% $Man_5GlcNAc_2$ produced *in vivo*.[2] This result is especially noteworthy because

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[2] These data can be provided in the form of an inventor declaration upon request by the Examiner.

Chiba discloses that using an Och1p targeting sequence with an *A. saitoi* mannosidase catalytic domain *fails* to produce $Man_5GlcNAc_2$ (page 26302, 2nd column, 1st paragraph).

Applicant's post-filing date publications also show that the amended claims are enabled. Choi *et al.*, 2003 (*Proc. Natl. Acad. Sci. U.S.A.* 100, 5022-5027) (co-authored by the inventor and performed according to the methods of the instant application; copy attached as **Exhibit C**), reports a combinatorial mannosidase library produced according to the disclosure of the instant application used to produce humanized glycoproteins in a lower eukaryotic host. Choi *et al.* shows glycans from strains engineered with targeted mannosidase I (an α-1,2-mannosidase) and demonstrate the production of "$Man_5GlcNAc_2$ that can serve as a substrate for GlcNAc transferase I *in vivo* and that is produced within the host cell in excess of 30%" (Figures 3D-F).

The efficacy of the instant invention is further demonstrated in Hamilton *et al.* (*Science* 301:1244-1246 (2003); copy attached as **Exhibit D**) which documents the production of $Man_5GlcNAc_2$ that is a substrate for GlcNAc transferase I *in vivo* in excess of 30% (see, e.g., Figure 2 which shows the production of $Man_5GlcNAc_2$ and its subsequent conversion to $GlcNAcMan_3GlcNAc_2$ by GnTI and Mannosidase II, followed by the conversion of $GlcNAcMan_3GlcNAc_2$ to $GlcNAc_2Man_3GlcNAc_2$ by GnTII).

The above-cited publications by the applicant and co-workers (Choi et al., 2003 and Hamilton et al., 2003) also show that chromosomal integration of an appropriate mannosidase fusion (typically resulting in a single gene copy) results in the desired outcome of more than 30% $Man_5GlcNAc_2$ *in vivo*. The original application teaches that any method

of transforming into the host cell nucleic acids encoding hybrid glycosylation enzymes may be used, including those which result in chromosomal integration (page 30, lines 18-28). As discussed above, the methods of the prior art, even when expressing hybrid mannosidase from high copy plasmids, were unable to achieve this level of $Man_5GlcNAc_2$ production *in vivo* so that the $Man_5GlcNAc_2$ so produced could act as substrate for GnTI and thereby feed into a human-like glycosylation pathway.

4. Conclusion

The application as originally filed teaches the skilled worker how to introduce stable mannosidase activity into the ER or Golgi of a host cell using the intracellular targeting methods and libraries of the invention, thereby producing a cell in which "in excess of 30 mole % of the N-glycan structures produced within the host cell have a $Man_5GlcNAc_2$ structure that can serve as a substrate for GlcNAc transferase I *in vivo*" as required by the claims as amended herein. The instant application shows clearly that applicant was in possession of the claimed invention at the time of filing. The original application clearly describes the instant invention using words, structures, figures,tables and examples to detail how one skilled in the art can introduce stable mannosidase activity (or any glycosylation activity) into the ER or Golgi of a host cell. Furthermore, post-filing date evidence establishes that, following the teachings of the instant application, the claimed invention can be clearly carried out by one skilled in the art without undue burden. Accordingly, applicant respectfully requests that the enablement rejections be withdrawn.

D. 35 U.S.C. § 102 Claim Rejections – Chiba

Claims 35-42, 46-48, 52, 54-56, 59 and 60 stand rejected under 35 U.S.C. 102(b) as being anticipated by Chiba et al., 1998 (*supra*; "Chiba"). According to the Examiner (Office Action, page 10), Chiba discloses a method for producing a human-like glycoprotein in *S. cerevisiae* host cells by producing fusion proteins from a vector comprising nucleic acids encoding an alpha-1,2 mannosidase linked to a ER signal peptide (HDEL), and further discloses doing so in a yeast strain lacking mannosyltransferase expression (OCH1, MNN1 and MNN4). And, according to the Examiner, Chiba discloses that the glycoproteins so produced are isolated from the host and further analyzed. The Examiner points out that, as the resulting yeast strain did produce the $Man_5GlcNAc_2$ carbohydrate structure, the expressed mannosidase "*is functional* at the pH at the target organelle" (Office Action, page 10; emphasis added). Applicant agrees in part with the Examiner. However, the claims as amended herein are patentable over Chiba, as described below.

The claimed invention requires not merely that $Man_5GlcNAc_2$ be produced (as in Chiba), but that it be produced (a) *in the right place* (i.e., *within the host cell* and not secreted); (b) in the *correct isomeric configuration* to be an active substrate for GlcNAc transferase I ("GnTI"; the next human-like glycosylation reaction); and (c) *in high enough amounts* (more than 30 mole % of the N-glycans) that a significant fraction of N-glycan intermediates are converted by GnTI to the next intermediate in a human-like glycosylation pathway.

The present invention provides methods and libraries which enable the skilled worker to engineer a host cell to produce human-like (i.e., "humanized") glycoproteins. Essential to the methods of the invention is that sufficient amounts of the $Man_5GlcNAc_2$ isomer that can act as a substrate for GnTI be generated so that a significant fraction of N-glycan intermediates are converted by GnTI to the next intermediate in a human-like glycosylation pathway. $Man_5GlcNAc_2$ is the precursor for five subsequent enzymatic reactions leading to a humanized glycoprotein ($Man_5GlcNAc_2$→ $GlcNAcMan_5GlcNAc_2$→ $GlcNAcMan_3GlcNAc_2$ → $GlcNAc_2Man_3GlcNAc_2$→ $Gal_2GlcNAc_2Man_3GlcNAc_2$ → $NANA_2Gal_2GlcNAc_2Man_3GlcNAc_2$). As each glycosylation reaction is typically incomplete, starting with a low amount (*i.e.*, less than 30%) of $Man_5GlcNAc_2$ will result in a miniscule amount of human-like glycoprotein ($NANA_2Gal_2GlcNAc_2Man_3GlcNAc_2$) in the end. The more $Man_5GlcNAc_2$ produced in the first step, the higher the yields of human-like glycoproteins produced in subsequent glycosylation steps in the host cell of choice.

Chiba shows only about a 10% total conversion of $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ glycans when a cloned α-1,2-mannosidase (fused to the HDEL targeting sequence) is expressed within a host cell (Chiba, Figure 6C, panel b, p. 26302). Further, Chiba shows that the $Man_5GlcNAc_2$ produced on a marker protein known to be retained *within* the host cell (CPY) is produced at a molar ratio of only 27% (Figure 6C, compare "M5" in panels a and b; molar ratios reported on p. 26302, left col., second paragraph). Nothing in Chiba, however, shows that the $Man_5GlcNAc_2$ intermediates produced were in the appropriate isomeric configuration to serve as substrates for GnTI, as required by the instant claims.

Chiba also reports making a hybrid in which the *A. saitoi* mannosidase domain is attached to the transmembrane domain of a Type II Golgi protein, Och1p (an early Golgi membrane protein). Notably, these host cells failed to produce $Man_5GlcNAc_2$ (Chiba, page 26302, right column, first paragraph). Chiba thus *teaches away* from using signal peptide sequences from Type II Golgi proteins -- and specifically from using an Och1p localization sequence -- to target a heterologous mannosidase catalytic domain in the glycosylation pathway of a lower eukaryotic host cell. As mentioned above, applicant has and can provide to the Examiner data showing production of $Man_5GlcNAc_2$ using the Och1p targeting sequence.

In summary, Chiba tried to produce human-like glycoproteins in yeast by taking a catalytic domain from an alpha-1,2 mannosidase (*A. saitoi*). This enzyme was expressed previously in yeast and found to be active upon preparation of cell free extracts from the transformed yeast cells (Inoue et al., 1995, *Biochim. Biophys. Acta,* 1253: 141-145). Chiba attempted to target this enzyme to the glycosylation pathway of the host cell (e.g., to the Golgi using an Och1p leader and to the ER using an "HDEL" retention/retrieval signal). None of these approaches succeeded in producing $Man_5GlcNAc_2$ on more than 27% of the N-glycans expressed in the engineered host cells – even when the hybrid mannosidase was expressed from a high copy number plasmid.

Many possible solutions could be envisioned to address the problems of Chiba's method, such as to increase expression levels further using stronger promoters or nucleic acid stabilization techniques. But nowhere did the prior art teach or suggest that the problem could be inefficiency in the activity of the catalytic domain where it was targeted,

e.g., because the pH optimum of the enzyme did not match that of the location to which it was targeted.

The instant invention provides such an improvement to Chiba's methods. Applicant teaches specifically how to overcome the deficiencies of the prior art by focusing on the choice of mannosidase (by considering the environment in which the enzyme is expected to act) and the targeting peptide used for its localization. Applicant's novel method for overcoming the inefficiency of previous approaches involves the construction of a combinatorial DNA library, useful for making and identifying optimized hybrid glycosylation enzymes comprising targeting sequences coupled to glycosylation enzyme domains (see, e.g., page 26, line 25 - page 30, line 16). Transformation of the DNA libraries into host cells and selection of cells from the libraries of the invention are discussed on pages 30 – 32. In one preferred embodiment, the DNA library of the invention is used to make and select a hybrid mannosidase enzyme that produces high yields of $Man_5GlcNAc_2$ structure (see, e.g., page 20, line 8 to page 25, line 20; page 33 (Example 1); page 37 (Table 6). Using the methods and libraries taught in the application as originally filed, a person skilled in the art can introduce stable mannosidase activity into the Golgi of a host cell, as recited by the amended claims, resulting in the production of $Man_5GlcNAc_2$ structures on greater than 30 mole % of the N-glycans produced in the host cell.

Chiba neither discloses or suggests that it is *the correct combination* of catalytic domains and targeting signal peptide sequences -- taking into consideration, e.g., the pH of the particular subcellular location in the host organism to which the catalytic domain is targeted -- that results in optimal mannosidase activity *in vivo*. As discussed above, Chiba

does not disclose the pH of the subcellular location to which the mannosidase is targeted, or the selection of a cellular signal peptide to target the catalytic domain to a location for optimal activity.

Nor does Chiba disclose nor suggest a hybrid mannosidase enzyme comprising a catalytic domain having a pH optimum within 1.4 pH units of the average pH optimum of other glycosylation enzymes in the subcellular location where the domain is targeted. Rather, Chiba used a mannosidase catalytic domain with a pH optimum of about 5.0, and targeted it to a subcellular location having other glycosylation enzymes with average pH optima of about 6.5 to 7.0, i.e., a difference greater than 1.4 pH units. Amended/added claims 35, 67 and 70 require that the mannosidse catalytic domain have a pH optimum closer to that of the other resident glycosylation enzymes.

Overall, the $Man_5GlcNAc_2$ structures that have been generated by prior art methods were produced at insufficient yields (*i.e.*, less than 30%) and not demonstrated to be a substrate for GnTI. Therefore, $Man_5GlcNAc_2$ glycan structures produced by prior art methods could not effectively be further "humanized" for the production of therapeutic proteins. Failure of such prior art methods and the surprising success of the methods and combinatorial genetic libraries of the present invention have been reviewed in several recent publications. By following the teachings of the present invention, applicant has been able to demonstrate not only the successful conversion of $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ *in vivo*, but also the conversion of $Man_5GlcNAc_2$ to $GlcNAcMan_5GlcNAc_2$ *in vivo* -- a step that had eluded previous researchers for over 10 years (Choi et al., 2003; **Exhibit C**).

In a recent Scientific American article in which the author reviews the applicant's scientific work which led to the instant invention, he compares the work of Kirin Brewery (authors of Chiba *et al.* 1998):

> The biggest challenge [after deleting certain yeast genes], and one that had foiled other investigators, came next: to create an assembly line of enzymes needed to put the appropriate sugars on a human protein manufactured in a yeast cell. ***Kirin Brewery, for one, had inserted the human gene for a critical glycosylation enzyme in yeast, but little had happened.*** The GlycoFi team [***led by applicant***] reasoned that for the enzyme to work, it would have to get to the right place in the yeast cell....This [GlycoFi's] sugar assembly line has functioned better than anyone expected.

G. Stix, *Scientific American* (January 2004), pgs. 32-33 at 33, left col. (emphasis added) (copy attached as **Exhibit E**).

Bretthauer (*TRENDS in Biotechnology*, 21:459-462 (2003) (copy attached as **Exhibit F**)) discusses the work of Chiba and other subsequent publications from researchers in this area (pgs. 459, rt. col.- page 461, left col.) and then states that the work of Choi *et al.* (**Exhibit C**; co-authored by applicant and performed according to the methods of the invention) "represents a major step in the 'humanization' of N-linked oligosaccharide pathways in fungal hosts" (page 461, left col.).

For all the above reasons, Chiba neither anticipates nor renders obvious applicant's claims as amended herein. Accordingly, applicant respectfully requests that the Examiner withdraw her 102(b) rejection.

Entry of this Amendment and allowance of the claims as submitted herewith is respectfully requested.

Respectfully submitted,

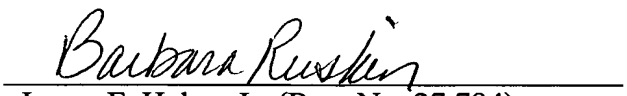

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EXHIBIT A

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Filamentous fungi as production organisms for glycoproteins of bio-medical interest

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Filamentous fungi are commonly used in the fermentation industry for large scale production of glycoproteins. Several of these proteins can be produced in concentrations up to 20–40 g per litre. The production of heterologous glycoproteins is at least one or two orders of magnitude lower but research is in progress to increase the production levels. In the past years the structure of protein-linked carbohydrates of a number of fungal proteins has been elucidated, showing the presence of oligo-mannosidic and high-mannose chains, sometimes with typical fungal modifications. A start has been made to engineer the glycosylation pathway in filamentous fungi to obtain strains that show a more mammalian-like type of glycosylation. This mini review aims to cover the current knowledge of glycosylation in filamentous fungi, and to show the possibilities to produce glycoproteins with these organisms with a more mammalian-like type of glycosylation for research purposes or pharmaceutical applications

Keywords: **filamentous fungi, homologous and heterologous protein secretion, N-glycosylation, O-glycosylation, phosphorylation, protein-linked carbohydrates.**

Introduction

The expression of human genes in a variety of heterologous expression systems has become an important technique to produce recombinant proteins for research purposes or pharmaceutical applications. It is generally recognised that there is no universal expression system available for (biopharmaceutical) production, and the selection of a cell type for expression of heterologous proteins depends on a number of criteria. One of the important criteria to be considered, is whether a protein needs to be (correctly) glycosylated for its application. Many human therapeutics are glycoproteins, and it is known that the glycosylation modulates numerous protein characteristics [1,2]. Glycoproteins that are not correctly glycosylated may be misfolded, biologically inactive, cleared from the circulation too fast, or exert an unwanted immunological response. Chinese hamster ovarian cell lines (CHO cells) have been shown to be suitable hosts for the expression of a number of sialylated glycoproteins (see for example [3,4]). The availability of several mutant CHO cell lines, stable transfected with additional glycosyltransferases, increases the potential of this system to "mimic" the natural glycosylation of specific glycoproteins.

For a number of glycoproteins, the presence of site-specific N-glycosylation is important, but the precise structure of the glycans apparently does not directly influence the biological function of the protein. Dependent on the application of the glycoprotein, it may be considered to use alternative eukaryotic expression systems for the production of such glycoproteins, e.g., insect cells, plants or filamentous fungi. Insect cells infected with recombinant baculovirus vectors already have become a popular expression system for the production of mammalian glycoproteins [5]. Recombinant glycoproteins expressed in this system mostly contain truncated paucimannosidic N-glycans [5]. In some cases the presence of complex-type glycans has been reported, both "wanted" glycans containing a LacNAc unit, and "unwanted" glycans containing the immunogenic core α 3-fucose epitope [6–9].

Filamentous fungi are natural secretors of a number of glycoproteins (mainly enzymes), often in abundant quantities, and are able to grow on relatively inexpensive media. Due to these properties and also to their GRAS (General Regarded As Safe) status the filamentous-fungal species *Aspergillus niger, Aspergillus awamori* and *Aspergillus oryzae* are widely used by the fermentation industry for the

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production of a great variety of extracellular enzymes mainly used in the food industry [10]. Also the filamentous fungus *Trichoderma reseei* is widely used for the production of enzymes used in the laundry and paper and pulp industry. Initially the production levels of these enzymes were relatively low, but by performing long-lasting classical strain-improvement programs, industrial production strains have been obtained by which high levels of fungal-enzyme (e.g., more than 20 g/l of *A. niger* glucoamylase and more than 40 g/l of *T. reesei* cellobiohydrolase I) are produced [11].

The application of modern molecular-genetic techniques has, however, greatly accelerated fungal strain improvement and has created new opportunities for the production of fungal enzymes, but also of non-fungal enzymes and pharmaceutical or diagnostic proteins. In the last years all the "tools" have been developed, which are required for (molecular)-genetic analysis of *A. niger, A. awamori, A. oryzae* and *T. reesei* and for construction of high production strains [12].

Filamentous fungi as host for the expression of heterologous proteins

To evaluate the potential of filamentous fungi to high level production of fungal and non-fungal glycoproteins by genetic modification, the overproduction of filamentous-fungal enzymes by *A.niger, A. oryzae* and *T. reesei* has been studied. The most common strategies by which high level production of fungal enzymes have been achieved are based on the use of well characterised strong, constitutive or regulated gene expression signals and the generation of multicopy strains. Such strains can be obtained using cosmid vectors, containing several gene-copies, and/or using selection markers which facilitate multicopy vector-integration. With these systems at least 10–20-fold improvement of most fungal enzymes could be achieved, resulting in the production of up to 1–2 gr/l in a shake flask culture (for a review see [12]). From these overexpression studies it also became clear that a limitation at the level of transcription is the main bottleneck for achieving higher production levels.

For the production of heterologous proteins of mammalian, bacterial, avian or plant origin, the expression systems that have been developed for the overproduction of fungal proteins, have been used. Unfortunately the initial production levels were much lower and with some exceptions reached levels that did not exceed a few milligrams per litre (see reviews [13–15]). Several empirical improvement studies and a limited number of systematic analysis-studies have provided evidence that the following factors negatively influence the initial production levels: i) mRNA-instability [16], ii) codon usage [17], iiii) inefficient translocation, folding and transport through the secretion pathway [18,19] and iii) extracellular degradation [20–22]. From the data available, it is clear that the limiting factors often depend on the protein to be expressed and/or the filamentous fungal host strain. On the basis of the identified factors that limited the efficient production of non-fungal proteins, several strategies have been developed for improved production (see reviews [19,23]). Most of these strategies are similar to those for fungal proteins [12] and comprise (i) the introduction of large number of copies, (ii) the use of efficient transcription, translation and secretion control-signals, (iii) the construction and use of protease-deficient host strains, (iv) development of optimal production media and (v) gene fusion with a gene encoding part or whole of a well-secreted protein. Especially the latter strategy has shown to be very effective resulting in an improvement varying from 5 to 1000-fold, depending on the protein, resulting in protein levels varying from 5 mg/l to 250 mg/l (see Table 1). Even higher production levels, up to 1–2 g/l, were obtained for the glycoproteins bovine chymosine [24] and lactoferrin [25] when high-level production strains were subjected to several rounds of mutagenesis and selection. Research is still in progress to further improve the production of glycoproteins by optimising the primary sequence of the genes of interest, improvement of the mRNA stability, overproduction of chaperones and foldases, and isolation of strains which are deficient in protease acting during the secretion process, in the vacuole and/or in the cultivation medium.

The structures of protein-linked carbohydrates from filamentous fungi

Similar as mammalian cells, filamentous fungi produce soluble and membrane-bound post-translationally modified proteins, that may carry N- and/or O-glycans. Filamentous fungi most often synthesise small high-mannose type N- and O-glycans [23]. Table 2 gives an overview of characterised glycans from several species of filamentous fungi. This overview shows a remarkable complexity in glycan structures. At the moment it is not clear which enzymes contribute to the formation of these oligosaccharides. Probably, a whole set of glycosyltransferases and glycosidases in the secretion apparatus are involved. In addition, filamentous fungi are known to secrete different glycosidases to the extracellular medium, that may trim protein-linked glycans [26–31]. The latter modifications may add to the observed heterogeneity of the glycans.

Sofar, no typical mammalian-like complex-type glycans have been found in filamentous fungi [32]. Some of the glycans found (see Table 2) resemble mammalian high-mannose glycans [Man(6-9)GlcNAc2]. In addition, typical "fungal-type" glycans have been identified on different *Aspergillus* and glycoproteins that are structurally different from the mammalian glycans [Man(5–12)GlcNAc2] and that are probably synthesised by not yet characterised fun-

Table 1. Production of heterologous proteins by filamentous fungi using a gene-fusion strategy

Protein and host	*"Carrier" gene*	*"Kex2" linker*[a]	*Protein level*	*Improvement factor*	*Ref.*
Bovine chymosine					
A. awamori	–	–	8 mg/l		[25]
	A. awamori glaA 1-614		140 mg/l	10–20 ×	[25]
Fab-antibody fragments					
T. reesei	–	–	0.3–1 mg/l		[82]
	T. reesei cbhI 1-466		5–40 mg/l	5–100	[82]
Hen egg-white lysozyme					
A. niger	–	–	50 mg/l		[13]
	A. niger glaA 1-498	+	1000 mg/l	>20 ×	[13]
Human Interleukin-6					
A.niger	–	–	nd[b]		[20]
	A. niger glaA 1–514	+	15 mg/l	>1000 ×	[20]
A. awamori	–		50 ug/l		[17]
	A. niger glaA 1-514		15 mg/l	300 ×	[17]
Human lactoferrin					
A. awamori	+	+	25 mg/l		[25]
	A. niger glaA 1–498	+	>250 mg/l	>10–50 ×	[25]
Single chain Fv anti-lysozyme					
A. niger	–	–	10–30 mg/l		[83]
	A. niger glaA 1-514	+	50–80 mg/l	2–6 ×	[83]
Human thrombomodulin					
A. chrysogenum	–	–	15 mg/l		[84]

[a]Cleavage occurs by a KEX2-like protease for which a recognition site had been introduced in the fusion protein; [b]nd: not detectable

gal mannosyltransferases in the Golgi compartment. Very small N-glycans such as Man(α1–2)GlcNAc2 were also found, for instance on *A. awamori* glucoamylase [33]. The genes of specific α-1,2-mannosidases from *T. reesei, A. saitoi* and *P. citrinum* have been cloned [34,35; Maras et al., submitted manuscript], but it is not clear whether these enzymes are actually involved in processing of N- and/or O-glycans.

In contrast to *Saccharomyces cerevisiae,* hyperglycosylation is not a typical feature for filamentous fungi. Hyperglysolation of proteins, however, has been detected in some strains of filamentous fungi [36] and it may be dependent on the growth conditions if an heterologous protein contains small-sized N-glycans or hyperglycosyl structures [37].

Recently, the presence of single, non-substituted GlcNAc residues has been detected on potential N-glycosylation sites of proteins derived from certain fungal species, such as *T. reesei* and *Aspergillus tubigensis* [38–40]. Until now the biosynthesis of this type of glycosylation is not clear. A deglycosylating enzyme, analogous to 'Endo H' could be responsible for the formation of these single N-acetylglucosamines, or the single GlcNAc could result from extensive trimming by several different exoglycosidases. Alternatively, transfer of GlcNAc to potential N-glycosylation sites is due to the action of a modified "oligosaccharyltransferase" [41].

In analogy with yeast, phosphate residues were detected on N-glycans of some filamentous fungi. A small amount of the carbohydrate portion of β-galactosidase from *A. oryzae* was found to consist of short high-mannose glycans containing phosphate in monoester linkage and galactomannan-type sugar chains with phosphate in mono- and diester linkages [42]. The presence of phosphate residues was also established on N-glycans of cellobiohydrolase I secreted by the filamentous fungus *T. reesei* [40,43]. Using a combination of different techniques among which homonuclear and heteronuclear NMR techniques, the structure of ManPGlcMan7GlcNAc2 was proposed (Figure 2(A) [43,44]. At the moment, the linkage positions of phosphate residues on N-glycans of most filamentous fungi have not been studied in enough detail to speculate on substrate specificities and functions of phosphorylating enzymes. It may be possible that the phosphorylation process resembles that of *S. cerevisiae* which has been studied more extensively and generally appears to occur both on α-1,2 and on α-1,6 linked Man α-1,2-Man (Figure 2B) [45–48]. Next to phosphorylation of N-glycans, phosphorylation of O-glycans has been reported to occur in yeast [49].

Table 2. An overview of partially or completely characterised oligosaccharides on glycoproteins from filamentous fungi

Species	*Studied glycoprotein*	*Number of glycans*	*N-glycan structure*	*O-glycan structure*	*Presence of phosphate*	*Reference*
A. oryzae	Taka amylase A	1 N	$Man_6GlcNAc_2$	–		[85]
	Coprinus cinereus peroxidase	1 N / 2 O	$Man_{(1-7)}GlcNAc_2$ single GlcNAc	$Man_{(1-5)}$	–	[86]
	β-galactosidase	4 N	96 % of short sugarchains including $Man_{(5-11)}GlcNAc_2$ 4 % hyperglycosyl (galactomannan)	–	+	[42]
	Glucoamylase			$Man_{(1-3)}$ GlcMan		[62]
A.saitoi	Acid carboxypeptidase		$Man_{(5-6)}GlcNAc_2$ $Man_{(8-12)}GlcNAc_2$	Man		[66]
A. niger	Endo-polygalacturonase	1 N	$Man_{(5-11)}GlcNAc_2$	–	–	[57]
	Glucose-oxidase		$Man_{(5-7)}GlcNAc_2$ + non-characterised short sugarchains	Man		[56]
	α-D-glucosidase		$Man_{(8-9)}GlcNAc_2$ $GlcMan_9GlcNAc_2$ $GalfMan_{(5-8)}GlcNAc_2$			[51]
	α-D-galactosidase		$Man_{(5-6)}GlcNAc_2$ $Man_9GlcNAc_2$ $GlcMan_9GlcNAc_2$ $GalfMan_4GlcNAc_2$ $GalfMan_5GlcNAc_2$			[50]
	Phytase		$Man_{(5-10)}GlcNAc_2$			[58]
	Glucoamylase	2 N/ 35 O		$Man_{(1-3)}$ GlcMan		[62]
A. nidulans	Invertase		Galactomannan (hyperglycosylation?)	–	+(?)	[55]
A.tubigensis	PNGase At	9N/1O	$Man_{(5-9)}GlcNAc_2$ single GlcNAc	$Man_{(1-2)}$		[38]
A. awamori	Glucoamylase	2 N/ 40 O	NB	$Man_{(1-2)}$ $GlcMan_2$ $GalMan_2$		[69]
Var. *Kawachi*	Glucoamylase		Galactomannaan	Glucosylated high-mannose glycans		[54]
	Glucoamylase		$Man_2GlcNAc_2$			[33]
T. reesei	Cellobiohydrolase I	3 N/ 8 O	$Man_5GlcNAc_2$ $Man_9GlcNAc_2$	$Man_{(1-4)}$		[59]
	Cellobiohydrolase I		single GlcNAc $Hex_{(5-11)}HexNAc_2$	$Man_{(1-3)}$ Sulphate addition	+	[40]
	Cellobiohydrolase I		single GlcNAc			[39]
	Cellobiohydrolase I		$Man_{(5-7)}GlcNAc_2$ $Glc(ManP)Man_7GlcNAc_2$		+	[43]
T. viride	Cellobiohydrolase C	? N/17 O	single GlcNac	$Man_{(1-5)}$ GlcMan GalMan		[61]

– =absent; + =present
Empty boxes indicate that data are lacking.

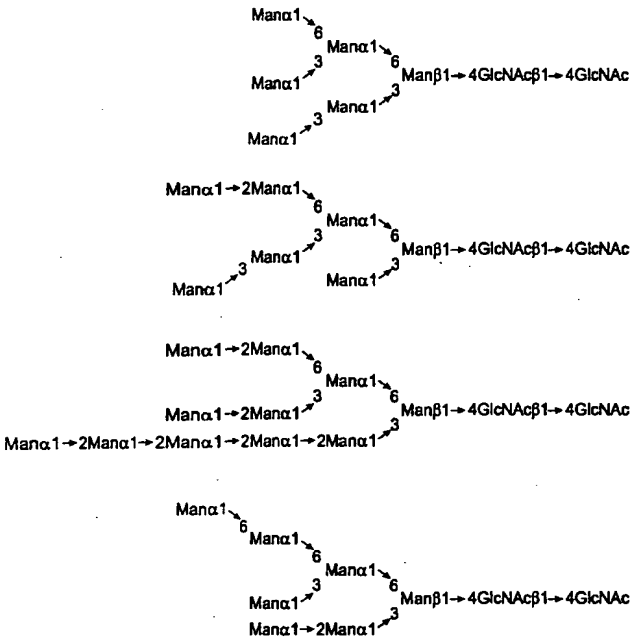

Figure 1. Fungi-type N-glycan structures. Mannose residues in red are probably incorporated by not yet characterised mannosyltransferases. The two upper oligosaccharides were found on glucose oxidase from *A. niger*, the third on acid carboxypeptidase from *A. saitoi* and the fourth glycan on β-galactosidase from *A. oryzae*.

Glucose and galactose residues have been detected at the non-reducing end of N-glycans of several species of filamentous fungi. Alfa-1,2-linked glucoses were found which probably resulted from inefficient trimming by glucosidase II. This lack of trimming has been observed with several species, and hence, it is a question whether this could be characteristic of filamentous fungi [43,51,52]. The presence of galactose residues was deduced from analyses of the monosaccharide compositions of N-glycans, but for some time it was difficult to assign their linkage positions. Groisman et al. [52] demonstrated that *Ascobolus furfuraceus* synthesised galactoses in the furanose form and not in the usual pyranose form. Alfa-linked galactofuranoses were detected on α-D-glucosidase and α-D-galactosidase from *A. niger* [50,51]. Beta-linked galactofuranose was detected on ascorbate oxidase from Acremonium sp. H1-25 [53]. The structures of these glycans were resolved by NMR analyses and are presented in Figure 3. Other research groups also mentioned galactomannans on glycoproteins from filamentous fungi, but without adding structural information [54,55]. The presence of these galactomannans seems to be strain dependent since other *A. niger* strains apparently do not transfer galactoses to their glycans [56–58]. Significant strain dependent differences were also revealed in N-glycan structures of *T. reesei* [39,40,43,59]. Growth conditions have been found to influence the glycan modification of some proteins, possibly by modulating the expression of enzymes involved in the fungal glycosylation pathway [60]. For these reasons it is important to mention the fungal strain and the exact growth conditions when glycan structures of a specific glycoprotein are reported.

The O-glycans found in filamentous fungi up till now differ from those found in higher eukaryotes [32]. Filamentous fungi synthesise O-glycans that vary from a single mannose to linear mannopentaoses. In some cases, the latter glycans were found to be substituted with glucose and/or galactose [40,61–63]. Harrisson et al. [40] suggested the presence of sulfate groups on O-glycans from *T. reesei*.

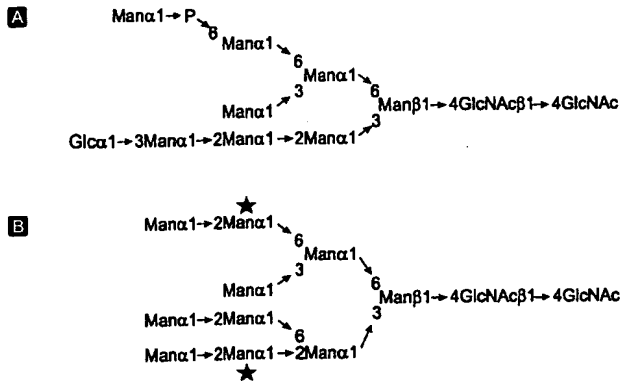

Figure 2A. The structure of a phosphorylated N-glycan on *T. reesei* Rut-C30 cellobiohydrolase I. **2B Core phosphorylation on *Saccharomyces cerevisiae* glycoproteins.** The positions where phosphate groups are added to the core portion of yeast N-glycans are marked with asterisks.

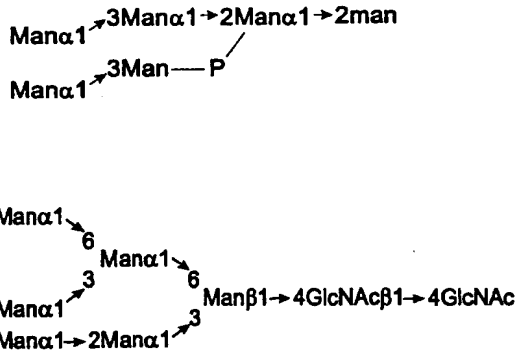

Figure 3. Structures of galactosylated fungal N-glycans. The above N-glycans were found on α-D-glucosidase from *A. niger*. The lower N-glycan structure was found on ascorbate oxidase from *Acremonium* sp. H1-25.

The importance of glycosylation for properties of homologous and heterologous proteins

Analogous to the mammalian system, protein-linked glycans are important for the biological activity of some fungal proteins. In most cases observed sofar, however, N-glycans do not seem to be very important for the biochemical properties of fungal proteins, although much still has to be learned. In order to illustrate the above statements, some examples are given.

The zygomycete fungus *Rhizomucor pusillus* secretes aspartic proteinase, an enzyme which is used as a milk coagulant in cheese factories [64]. Production of this proteinase as a heterologous protein in *A. oryzae* resulted in increased mannosylation of the proteinase compared to the natural product. The recombinant proteinase showed a higher proteolytic activity and appeared to be less effective in milk-clotting. Deglycosylation by glycosidase or by amino acid replacement again led to a decrease of the first activity and an increase of the latter [65], indicating a functional importance of the glycan composition for the biological activity of this proteinase.

Aspergillus saitoi is known to secrete several extracellular proteinases, such as acid carboxypeptidase. Chiba et al. [66] showed that no extracellular carboxypeptidase could be found after tunicamycin treatment of *A. saitoi* cells, from which they concluded that N-glycosylation is important for secretion of this enzyme. Glycosylation may be also important for the secretion of heterologous proteins in fungi. In some cases the yield of heterologous protein produced in filamentous fungi is very low, which could be due (partly) to ineffective glycosylation. Morkeberg et al. [67] reported that overproduction of α-amylase in *Aspergillus oryzae* resulted in non-glycosylated product. An explanation could be that the glycosylation system cannot catch up with the high production rate. With α-amylase the glycosylation deficiency did not affect the production rate. For some heterologous proteins, however, glycosylation may be essential to obtain the proper conformation and high secretion levels. For example, the production of tissue plasminogen activator (tpA) as a well secreted product in Trichoderma has not been successful until now. Jarvis and Summers [68] demonstrated that secretion of tPA from insect cells is impaired when N-glycosylation is prevented. Similarly, inefficient glycosylation could be one of the factors responsible for the inefficient production of tPA in Trichoderma.

Neustroev et al. [69] showed that trimming of the O-glycans of *A. awamori* glucoamylase with α-mannosidase resulted in a decreased thermal and pH stability of the protein, indicating that O-glycosylation can confer thermostability to a protein. Goto et al. [36] described expression of a hyperglycosylated form of glucoamylase in *A. awamori var. kawachi*. This modified glucoamylase possessed higher digestibility of raw starch and higher stabilities in response to heat and extreme pH. Goto et al. [63] showed that O-glycans are involved in digestion by glucoamylase of raw starch through interaction with water, suggesting that O-glycans can be involved in hydration of substrate molecules. In a 'water-cluster-dissociating model' they proposed the oligomannose sugar chains to dissociate water clusters so that hydration of starch micelles with activated water molecules became possible. Partial replacement of mannose residues by glucoses led to a significantly decreased digestion of raw starch.

Absence of glycans can result in exposure of peptide fragments of a protein that are otherwise masked. As a result of this, aggregation can occur, the heterologous protein can be 'trapped' by interaction with an intracellular fungal protein or the protein is accessible to proteases and may be degraded [70,71].

Engineering of the glycosylation pathway in filamentous fungi

The N-glycans which are added to secreted proteins in filamentous fungi are dissimilar to those that are synthesised in mammalian cells. At the moment this is an important drawback for the application of fungi derived glycoproteins for therapeutic use [43,72,73]. Since compared to higher eukaryotes filamentous fungi are very easy to genetically modify, it is feasible to construct "lack of function" mutants that are disturbed in specific unwanted glycosylation steps, provided that the glycosylation pathway and the genes encoding the enzymes involved, are known. In contrast to yeasts, filamentous fungi are capable of extensive trimming of their N-glycans. A few fungal strains have been found that preferentially form Man5GlcNAc2 glycans [Maras and Contreras, unpublished results]. Furthermore, by selection of specific strains, in combination with mutagenesis it seems feasible to obtain strains that do not include phosphate groups in their glycans, and have lost the ability to hyperglycosylate, but instead mainly synthesise the Man5GlcNAc2 glycan that is the preferred starting glycan for complex-type glycosylation [74]. Subsequent engineering steps, i.e., introduction of the required mammalian glycosyltransferases may theoretically lead to strains that are able to accomplish complex-type glycosylation. The latter steps will be more complicated to accomplish, however, as the expressed heterologous glycosyltransferases have to be localised in specific membrane compartments, their activity levels should be adequate, and the donor substrates should be available in the fungi. Especially the last step, i.e. sialylation might prove difficult, as up till now no evidence is present that the donor substrate, CMP-neuraminic acid, is present in fungi. However, it may be possible to add *in vitro* sialic acid to recombinant glycoproteins by enzymatic synthesis. Recently it has been demonstrated that at least the first step towards a complex-type glycosylation, i.e. introduction of mammalian N-acetylglucosaminyltransferase I (GlcNAc-

T1) in filamentous fungi, has been accomplished. The cDNA encoding rabbit GlcNAc-TI has been introduced in the genome of *A. nidulans*. Kalsner *et al.* [75] demonstrated production of active enzyme by the fungus, but no evidence was presented for the *in vivo* transfer of GlcNAc residues to fungal glycans. In *T. reesei*, however, this *in vivo* transfer step has indeed been demonstrated. Structural elucidation by NMR of N-glycans of a strain, transformed with the cDNA encoding human GlcNAc-TI, established the formation of GlcNAcMan5GlcNAc2 in *T. reesei* [Maras et al., manuscript in preparation]. This result emphasises the fact that conversion of the glycosylation synthesis pathway is possible. However, the efficiency is not yet optimal and needs to be improved.

To take advantage of the high capacity of filamentous fungi for protein secretion, the glycosylation process should not only lead to correct mammalian-like glycans, but should also be stimulated to an elevated level to keep up with the high protein production to provide fully glycosylated and secreted glycoproteins.

Until now, synthesis of N- and O-glycans in filamentous fungi is very poorly studied. Since so little is known about glycan processing in filamentous fungi, the well studied pathway of the yeast *S. cerevisiae* is often used as a reference [76]. In fungi, only some steps of the dolichol-phosphate cycle for synthesis of the common Glc3Man9GlcNAc2 precursor glycan have been studied. Probably, this part of the N-glycosylation pathway is analogous to that in yeast. Palamarczyk et al. [32] mentioned that glycosylation can be enhanced by making available more of the lipid intermediates of the 'dolichol phosphate cycle'. Raising the temperature for growth of fungal cells for instance influences the dolichol content in ER membranes [77]. At elevated temperatures, dolichol kinase activity is stimulated. By adding Tween 80 or choline to the growth medium, an *in vivo* enhancement of mannose phosphate dolichol synthase (= MPD synthase) activity has been observed.

Some genes of this 'dolichol-phosphate cycle' have been cloned, amongst them dolichol phosphoryl mannose synthase from *Ustilago maydis* [78] and GTP:α-D-mannose-1-phosphate guanyltransferase from *T. reesei* [32]. Kruszewska *et al.* [79] overexpressed *S. cerevisiae* MPD synthase in Trichoderma and demonstrated enhanced cellulase secretion. It is known that in higher eucaryotes, terminal galactose residues on glycoproteins are recognized by the asialoglycoprotein receptor, resulting in the removal of these proteins from the circulation [80]. To convert fungi-like glycans to sialylated glycans, some "shortcuts" are proposed, which result in non-natural glycoproteins. Berg et al. [81] suggest to remove *in vitro* the major part of the glycan trees using an endo-N-acetylglucosaminidase. Depending on the enzyme used (EndoH or EndoF), the innermost one or two core GlcNAc residues are left on the protein. These residues are the acceptor for the successive action of a β-1,4[3]-galactosyltransferase and a sialytransferase, resulting in a typical sialylated lactosamine outer-chain. The endo-glycanase reaction is performed on native proteins. However, in such conditions not all glycans may be accessible to the enzyme and thus, some recognition determinants for mammalian receptors may still be present.

Conclusions

Filamentous fungi offer a very attractive, safe and cheap expression system for high level production of proteins. In contrast to yeast, they have a well developed secretion pathway, and naturally secrete high levels of proteins. At this moment, the therapeutic use of glycoproteins produced in filamentous fungi is very limited, as the glycans formed are dissimilar from mammalian glycans. However, specific filamentous fungal strains are available that can produce glycoproteins with short oligo mannose-type glycans. More knowledge and insight in the glycosylation pathway is needed to be able to engineer the glycosylation pathway to mimick a mammalian type of glycosylation. The prospects, however, to succesfully accomplish such an engineering by combined "lack of function" mutagenesis to prevent unwanted glycan epitopes and a "gain of function" engineering to add specific monosacharides to the glycan, are good.

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EXHIBIT B

EXHIBIT B: FROM GFI-100 CIP (10/371,877)

Example 16: Engineering *K.lactis* Cells to Produce *N*-glycans with the Structure $Man_5GlcNAc_2$

Identification and Disruption of the *K.lactis OCH1* gene

[0001] The *OCH1* gene of the budding yeast *S.cerevisiae* encodes a 1,6-mannosyltransferase that is responsible for the first Golgi localized mannose addition to the $Man_8GlcNAc_2$ *N*-glycan structure on secreted proteins (Nakayama et al, *J Biol Chem.*; 268(35):26338-45 (Dec 15, 1993)). This mannose transfer is generally recognized as the key initial step in the fungal specific polymannosylation of *N*-glycan structures (Nakanishi-Shindo et al, 1993; Nakayama et al, 1992; Morin-Ganet et al, *Traffic* 1(1):56-68. (Jan 2000)). Deletion of this gene in *S.cerevisiae* results in a significantly shorter *N*-glycan structure that does not include this typical polymannosylation or a growth defect at elevated temperatures (Nakayama et al, *EMBO J.*;11(7):2511-9 (Jul 1992)).

[0002] The *Och1p* sequence from *S.cerevisiae* was aligned with known homologs from *Candida albicans* (Genbank accession # AAL49987), and *P.pastoris* (B.K. Choi et al. in prep) along with the *Hoc1* proteins of *S.cerevisiae* (Neiman et al, *Genetics*, 145(3):637-45 (Mar 1997) and *K.lactis* (PENDANT EST database) which are related but distinct mannosyltransferases. Regions of high homology that were in common among *Och1p* homologs but distinct from the *Hoc1p* homologs were used to design pairs of degenerate primers that were directed against genomic DNA from the *K.lactis* strain MG1/2 (Bianchi et al, *Current Genetics* 12, 185-192 (1987)). PCR amplification with primers RCD33 (CCAGAAGAATTCAATTYTGYCARTGG) (SEQ ID NO:34) and RCD34 (CAGTGAAAATACCTGGNCCNGTCCA) (SEQ ID NO:35) resulted in a 302 bp product that was cloned and sequenced and the predicted translation was shown to have a high degree of homology to *Och1* proteins (>55% to *S.cerevisiae Och1p*).

[0003] The 302 bp PCR product was used to probe a Southern blot of genomic DNA from *K.lactis* strain (MG1/2) with high stringency (Sambrook et al, 1989). Hybridization was observed in a pattern consistent with a single gene indicating that this 302 bp segment corresponds to a portion of the *K.lactis* genome and *K.lactis* (*KlOCH1*) contains a single copy of the gene. To clone the entire *KlOCH1* gene, the Southern blot was used to map the

genomic locus. Accordingly, a 5.2 kb *Bam*HI/*Pst*I fragment was cloned by digesting genomic DNA and ligating those fragments in the range of 5.2 kb into pUC19 (New England Biolabs, Beverly, MA) to create a *K.lactis* subgenomic library. This subgenomic library was transformed into *E. coli* and several hundred clones were tested by colony PCR using RCD 33/34. The 5.2 kb clone containing the predicted *KlOCH1* gene was sequenced and an open reading frame of 1362 bp encoding a predicted protein that is 46.5% identical to the *S.cerevisiae OCH1* gene. The 5.2 kb sequence was used to make primers for construction of an *och1::KAN*^{R} deletion allele using a PCR overlap method (Davidson et al, *Microbiology*. 148(Pt 8):2607-15. Aug 2002). This deletion allele was transformed into two *K.lactis* strains and G418 resistant colonies selected. These colonies were screened by both PCR and for temperature sensitivity to obtain a strain deleted for the *OCH1* ORF. The results of the experiment show strains which reveal a mutant PCR pattern, which were characterized by analysis of growth at various temperatures and *N*-glycan carbohydrate analysis of secreted and cell wall proteins following PNGase digestion. The *och1* mutation conferred a temperature sensitivity which allowed strains to grow at 30°C but not at 35°C. **Fig. 12A** shows a MALDI-TOF analysis of a wild type *K.lactis* strain producing N-glycans of $Man_8GlcNAc_2$ **[c]** and higher.

EXHIBIT C

Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*

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The secretory pathway of *Pichia pastoris* was genetically re-engineered to perform sequential glycosylation reactions that mimic early processing of N-glycans in humans and other higher mammals. After eliminating nonhuman glycosylation by deleting the initiating α-1,6-mannosyltransferase gene from *P. pastoris*, several combinatorial genetic libraries were constructed to localize active α-1,2-mannosidase and human β-1,2-*N*-acetylglucosaminyltransferase I (GnTI) in the secretory pathway. First, >32 N-terminal leader sequences of fungal type II membrane proteins were cloned to generate a leader library. Two additional libraries encoding catalytic domains of α-1,2-mannosidases and GnTI from mammals, insects, amphibians, worms, and fungi were cloned to generate catalytic domain libraries. In-frame fusions of the respective leader and catalytic domain libraries resulted in several hundred chimeric fusions of fungal targeting domains and catalytic domains. Although the majority of strains transformed with the mannosidase/leader library displayed only modest *in vivo* [i.e., low levels of mannose $(Man)_5$-$(GlcNAc)_2$] activity, we were able to isolate several yeast strains that produce almost homogenous N-glycans of the $(Man)_5$-$(GlcNAc)_2$ type. Transformation of these strains with a UDP-GlcNAc transporter and screening of a GnTI leader fusion library allowed for the isolation of strains that produce GlcNAc-$(Man)_5$-$(GlcNAc)_2$ in high yield. Recombinant expression of a human reporter protein in these engineered strains led to the formation of a glycoprotein with GlcNAc-$(Man)_5$-$(GlcNAc)_2$ as the primary N-glycan. Here we report a yeast able to synthesize hybrid glycans in high yield and open the door for engineering yeast to perform complex human-like glycosylation.

The number of protein-based therapeutics entering preclinical and clinical evaluation has shown robust growth and is expected to increase in the years to come. Fueled by advances in proteomics and genomics as well as the ability to engineer and humanize monoclonal antibodies, protein-based therapeutics constitute ≈500 candidates currently in clinical trials (1). Several therapeutic proteins can be made in a prokaryotic expression system such as *Escherichia coli* (e.g., insulin); however, the majority of therapeutic proteins require additional posttranslational modifications to attain full biological function. N-glycosylation in particular is essential for proper folding, pharmacokinetic stability, and efficacy for a large number of proteins (2). Most therapeutically relevant glycoproteins, including antibodies, are therefore expressed in mammalian cells. However, volumetric productivity, product heterogeneity, media cost, retroviral contamination, and the time required to generate stable cell lines are generally viewed as drawbacks of mammalian cell culture.

Fungal protein-expression systems do not suffer from the same limitations, and protein titers of 14.8 and 35 g/liter have been reported for secreted heterologous proteins in yeast and the filamentous fungus *Trichoderma reesei*, respectively (3, 4). However, glycoproteins derived from fungal expression systems contain nonhuman N-glycans of the high mannose (Man) type, which are immunogenic in humans and thus of limited therapeutic value (5).

Fungi and mammals share initial steps of protein N-glycosylation, which involves the site-specific transfer of $(Glc)_3$-$(Man)_9$-$(GlcNAc)_2$ from the luminal side of the endoplasmic reticulum (ER) to the *de novo* synthesized protein by an oligosaccharyltransferase complex. Subsequent trimming by glucosidases I and II and a specific ER-residing α-1,2-mannosidase leads to the formation of a $(Man)_8$-$(GlcNAc)_2$ structures (isomer Man8B) (Fig. 1), the N-glycan found on most glycoproteins leaving the ER.

After the export of predominantly $(Man)_8$-$(GlcNAc)_2$ containing glycoproteins to the Golgi, the pathways diverge notably between mammals and yeast (6). In the human Golgi α-1,2-mannosidases (1A–1C) remove Man to yield the $(Man)_5$-$(GlcNAc)_2$ structure, which forms the precursor for complex N-glycans (Fig. 1*A*). These mannosidases are typically type II membrane proteins with an N-terminal cytosolic tail, a transmembrane domain, a stem region, and a C-terminal catalytic domain (Fig. 1*B*). Localization of these proteins, as with most enzymes involved in Golgi glycosylation, is mediated by the cytosolic tail, the transmembrane region, and the stem (7).

In *Saccharomyces cerevisiae*, N-glycosylation has been studied extensively and, unlike mammalian N-glycan processing, involves the addition of numerous Man sugars throughout the entire Golgi, often leading to hypermannosylated N-glycan structures with >100 Man residues. This process is initiated in the early Golgi by an α-1,6-mannosyltransferase (Och1p) that prefers $(Man)_8$-$(GlcNAc)_2$ as a substrate but is able to recognize various other Man oligomers with the notable exception of the human $(Man)_5$-$(GlcNAc)_2$ intermediate, which is not a substrate (8). After addition of this first α-1,6-Man by Och1p, additional α-1,6-mannosyltransferases will extend the α-1,6 chain, which then becomes the substrate for medial- and trans-Golgi-residing α-1,2- and α-1,3-mannosyltransferases as well as phosphomannosyltransferases that add yet more Man sugars to the growing N-glycan structure (9). In *Pichia pastoris* a very similar process occurs; however, hypermannosylation occurs less frequently and to a lower extent. In addition α-1,3-mannosyltransferase activity has not been found in this yeast, and N-glycans from *P. pastoris* do not have α-1,3-Man attached to the outer Man chain (10).

Humanizing the glycosylation machinery of a yeast strain will require the (*i*) elimination of some endogenous glycosylation reactions and (*ii*) the recreation of the sequential nature of human glycosylation in the ER and Golgi. Although the first step involves the generation of gene knockouts (e.g., α-1,6- and/or α-1,3-mannosyltransferases), the second step requires the proper localization of active mannosidases, glycosyltransferases, and possibly nucleotide sugar transporters to specific organelles. Moreover the formation of certain sugar–nucleotide precursor pools such as CMP-sialic acid may have to be engineered into the yeast host. Much is known about the localization of endogenous proteins in the secretory pathway of *S. cerevisiae* and other yeasts; however, there is no reliable method to predict whether a Golgi protein from one

Abbreviations: Man, mannose; ER, endoplasmic reticulum; GnTI, β-1,2-*N*-acetylglucosaminyltransferase I; MALDI, matrix-assisted laser desorption ionization; K3, Kringle 3 domain of human plasminogen; TOF, time of flight.

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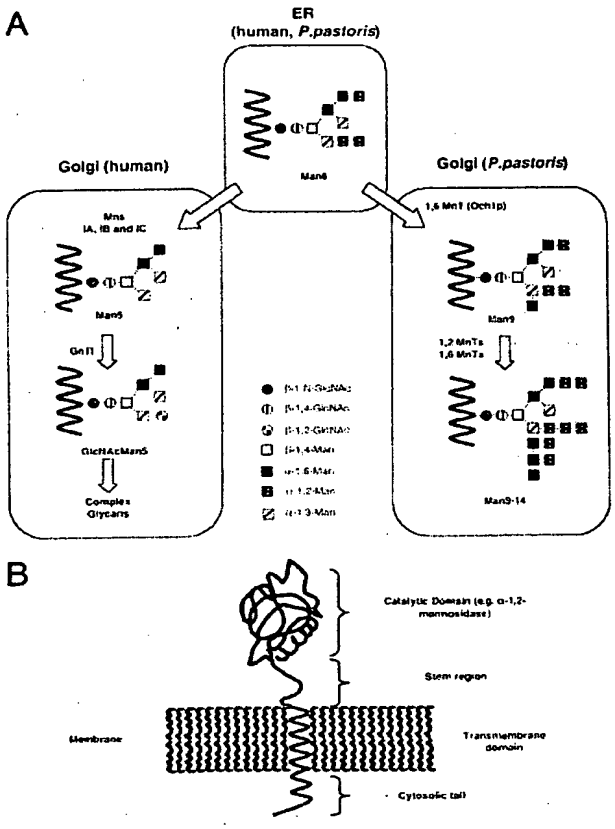


Fig. 1. (*A*) N-linked glycosylation pathway in humans and *P. pastoris*. Mns, α-1,2-mannosidase; MnT, mannosyltransferase; Och1p, the initiating 1,6-mannosyltransferase. (*B*) Structure of typical type II membrane glycosidase or glycosyltransferase.

organism will be properly localized in the Golgi of another organism. It is also unknown whether artificial in-frame protein fusions consisting of a yeast localization sequence and a catalytic domain from a nonyeast source (e.g., glycosyltransferases or mannosidases) will (*i*) localize to the desired organelle and (*ii*) show sufficiently high activity in the targeted environment. Although proper localization is important, it is not sufficient. Because the activity of the catalytic domain has to be maintained in the environment in which it has been localized, additional aspects such as pH optima must be considered. For example, Chiba *et al.* (11) found that localizing a mannosidase with a pH optimum of 5 in the ER of *S. cerevisiae* results in only modest intracellular mannosidase activity.

To overcome these major constraints we developed two tools: a combinatorial genetic library, generating hundreds of fusion constructs at a time, and a high-throughput screen that allows us to analyze large numbers of strains in parallel for their ability to modify N-glycans of recombinant reporter proteins. The combinatorial genetic library consists of an array of different fusion-protein constructs, each of which contains a fungal cellular targeting sequence fused in frame to a catalytic domain (e.g., mannosidase). Each of the 608 generated mannosidase fusion constructs was tested individually for its ability to catalyze the trimming of higher Man structures to $(Man)_5$-$(GlcNAc)_2$. Those strains that were able to generate mostly $(Man)_5$-$(GlcNAc)_2$ on a secreted reporter protein were subjected first to a second screen to ensure that trimming occurred *in vivo* and then engineered further to generate GlcNAc-$(Man)_5$-$(GlcNAc)_2$ by screening a similar β-1,2-*N*-acetylglucosaminyltransferase I (GnTI)/leader library. A more comprehensive article describing the characteristics of >600 leader/mannosidase and leader/GnTI fusions is in preparation (B.-K.C., P.B., R.C.D., S.R.H., A. Stadheim, H.L., R.G.M., J.H.N., S.W., and T.U.G., unpublished data); however, some of the most important findings are reported in this article.

Here we report the re-engineering of the secretory pathway in the methylotrophic yeast *P. pastoris*. The engineered strain produces predominantly N-glycans that are intermediates of the human glycosylation pathway, essentially void of fungal features. Our results suggest that further implementation of the described combinatorial library approach will allow for the engineering of yeast strains with increasingly human N-glycosylation. This article reports a genetically engineered yeast capable of producing a glycoprotein with a human-like hybrid N-glycosylation structure.

Materials and Methods

Strains, Culture Conditions, and Reagents. *E. coli* strains TOP10 or DH5α were used for recombinant DNA work. *P. pastoris* GS115 (*his4*, Invitrogen) or JC308 (*ura3*, *ade1*, *arg4*, *his4*, a gift from James M. Cregg, Keck Graduate Institute, Claremont, CA) were used for generation of yeast strains. Protein expression was carried out at room temperature in a 96-well-plate format with buffered glycerol-complex medium (BMGY) consisting of 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer (pH 6.0), 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% glycerol as a growth medium. The induction medium was buffered methanol-complex medium (BMMY) consisting of 1.5% methanol instead of glycerol in BMGY. Minimal medium is 1.4% yeast nitrogen base, 2% dextrose, 1.5% agar, and 4×10^{-5}% biotin and amino acids supplemented as appropriate. Restriction and modification enzymes were from New England BioLabs. Oligonucleotides were obtained from the Dartmouth College Core facility (Hanover, NH) or Integrated DNA Technologies (Coralville, IA). The enzymes, peptide *N*-glycosidase F, mannosidases, and oligosaccharides were obtained from Glyko (San Rafael, CA). Metal chelating HisBind resin was from Novagen. Lysate-clearing plates (96-well) were from Promega. Protein-binding 96-well plates were from Millipore. Salts and buffering agents were from Sigma. Matrix-assisted laser desorption ionization (MALDI) matrices were from Aldrich.

Cloning and Deletion of the *P. pastoris OCH1* Gene. The 1,215-bp ORF of the *P. pastoris OCH1* gene encoding a putative α-1,6-mannosyltransferase was amplified from *P. pastoris* genomic DNA (strain X-33, Invitrogen) by using the oligonucleotides 5′-ATGGCGAAGGCAGATGGCAGT-3′ and 5′-TTAGTC-CTTCCAACTTCCTTC-3′, which were designed based on the *P. pastoris OCH1* sequence (12). Subsequently, 2,685 bp upstream and 1,175 bp downstream of the ORF of the *OCH1* gene were amplified from a *P. pastoris* genomic DNA library (gift from Judah Folkman, Harvard Medical School, Boston) by using the internal oligonucleotides 5′-ATGGCGAAGGCAGATG-GCAGT-3′ and 5′-ACTGCCATCTGCCTTCGCCAT-3′ in the *OCH1* gene with T3 (5′-AATTAACCCTCACTAAAGGG-3′) and T7 (5′-GTAATACGACTCACTATAGGGC-3′) in the backbone of the library bearing plasmid λ ZAP II (Stratagene). The resulting 5,075-bp fragment was cloned into the pCR2.1-TOPO vector (Invitrogen) and designated pBK9. To create an *och1* knockout strain containing multiple auxotrophic markers, 100 μg of pJN329, a plasmid containing an *och1*::*URA3* mutant allele (J.F.N., S.W., and T.U.G., unpublished data; see Fig. 2*A* for details) was digested with *Sfi*I and used to transform *P. pastoris* strain JC308 by electroporation. After incubation on defined medium lacking uracil for 10 days at room temperature, 1,000 colonies were picked and restreaked. URA^+ clones that

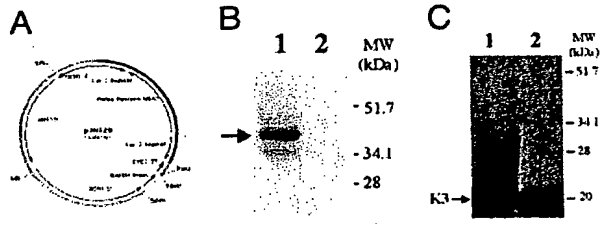


Fig. 2. Knockout of *OCH1* in *P. pastoris*. (*A*) The *OCH1* knockout plasmid pJN329 contains the *P. pastoris URA3* gene flanked by LacZ repeats and 2,878 bp upstream (5') and 1,011 bp downstream (3') of the *OCH1* gene of *P. pastoris*. (*B*) Immunoblot of Och1p in *Pichia* wild type (JC308, lane 1) and *och1* mutant (BK64-1, lane 2). The same amount of cell-free extract (50 μg per lane) was used, and Och1p was detected by using an Och1p peptide antibody followed by ECL. (*C*) The reporter protein K3 was expressed in *Pichia* wild type BK64 (lane 1) and an *och1* mutant, *P. pastoris* BK64-1 (lane 2). K3 was purified by Ni-affinity chromatography, separated by SDS/PAGE (4–20% gradient) under reducing conditions, and visualized by silver staining. The same amount of K3 (200 ng per lane) was loaded.

were unable to grow at 37°C but grew at room temperature were subjected to colony PCR to test for the correct integration of the *och1*::*URA3* mutant allele. One clone that exhibited the expected PCR pattern was designated YJN153.

Reporter Protein-Expression Construct. The Kringle 3 domain of human plasminogen (K3) was used as a model protein. A DNA fragment encoding the K3 (gift from Nick Menhart, Illinois Institute of Technology, Chicago) was amplified by using *Pfu* turbo polymerase (Stratagene) and cloned into *Eco*RI and *Xba*I sites of pPICZαA (Invitrogen), resulting in a C-terminal 6-His tag. To improve the N-linked glycosylation efficiency of K3 (13), Pro-46 was replaced with Ser-46 by using site-directed mutagenesis. The resulting plasmid was designated pBK64. The correct sequence of the PCR construct was confirmed by DNA sequencing.

Integration vectors and fusion constructs were generated based on the roll-in plasmids described by Lin Cereghino and coworkers (14); see details in *Supporting Methods*, which is published as supporting information on the PNAS web site, www.pnas.org.

Generation of Yeast Strains. To create *P. pastoris* strains expressing the K3, plasmid pBK64 was transformed into strains GS115 and YJN153, and colonies were selected on YPD medium containing 100 μg/ml zeocin to create strains BK64 and BK64-1, respectively. Plasmid pPB103 was linearized with *Eco*NI and transformed into strain BK64-1, and colonies were selected on defined medium lacking adenine. One strain containing the *Kluyveromyces lactis MNN2-2* gene was designated PBP1. Plasmids pBB27 and pBC4 were linearized with *Sal*I and transformed into strain PBP1, and colonies were selected on defined medium lacking histidine. Strains that were confirmed to contain the *Caenorhabditis elegans* α-1,2-mannosidase IB BB27 and BC4 fusion constructs were designated YJN188 and YJN168, respectively. Plasmid pNA15 was linearized with *Aat*II and transformed into strain YJN168, and colonies were selected on minimal medium without amino acids. One strain that was confirmed to contain the human GnTI gene fusion was designated YJN201.

Western Blotting. Proteins were separated by 4–20% gradient SDS/PAGE according to Laemmli (15) and then electroblotted onto nitrocellulose membrane (Schleicher & Schuell) as described (16). *P. pastoris* Och1p was detected by using an antibody raised against the peptide CQQLSSPKIDYDPLTL (Sigma–Genosys) with an ECL kit (Amersham Pharmacia).

Protein Purification. K3 was purified from the medium by Ni-affinity chromatography by using a 96-well format on a Beckman BioMek 2000 laboratory robot. The robotic purification is an adaptation of the protocol provided by Novagen for their HisBind resin.

Release of N-Linked Glycans. The glycans were released and separated from the glycoproteins by a modification of a previously reported method (17). After the proteins were reduced and carboxymethylated and the membranes were blocked, the wells were washed three time with water. The protein was deglycosylated by the addition of 30 μl of 10 mM NH_4HCO_3 (pH 8.3) containing 1 milliunit of *N*-glycanase (Glyko). After 16 h at 37°C, the solution containing the glycans was removed by centrifugation and evaporated to dryness.

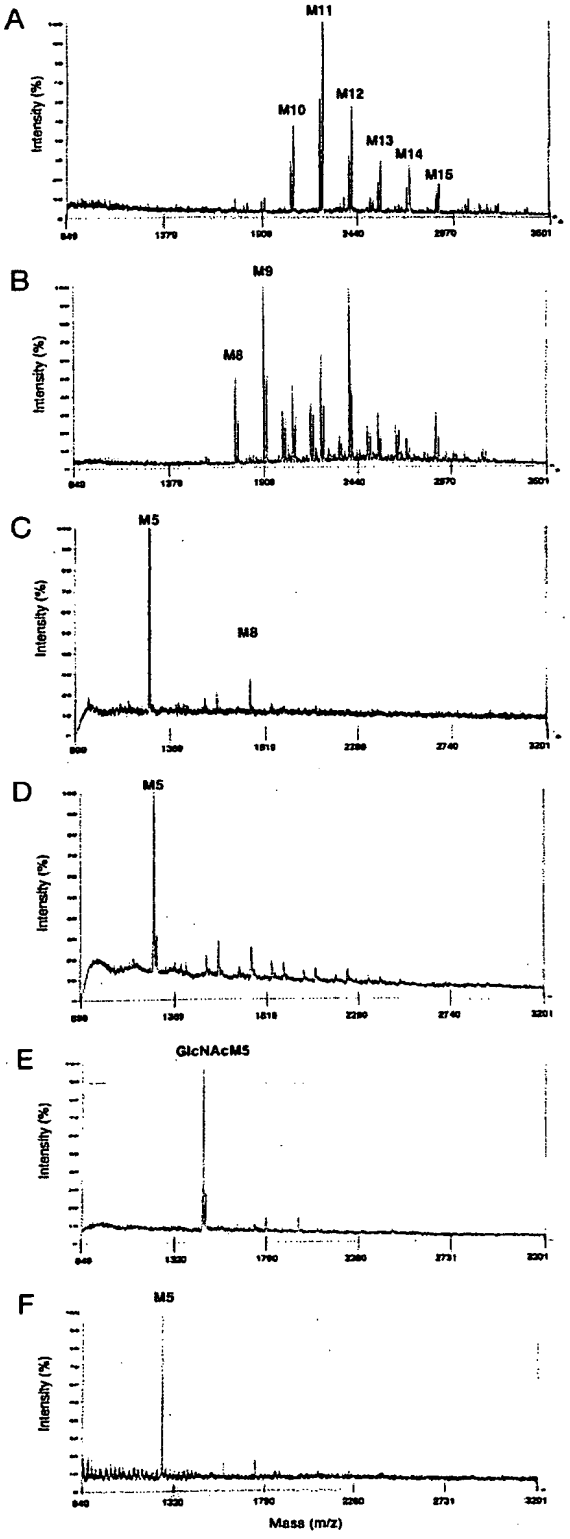
MALDI/Time-of-Flight (TOF) Mass Spectrometry. Molecular weights of the glycans were determined by using a Voyager DE PRO linear MALDI/TOF (Applied Biosciences) mass spectrometer with delayed extraction. The dried glycans from each well were dissolved in 15 μl of water, and 0.5 μl was spotted on stainless-steel sample plates and mixed with 0.5 μl of S-DHB matrix (9 mg/ml of dihydroxybenzoic acid/1 mg/ml of 5-methoxysalicylic acid in 1:1 water/acetonitrile/0.1% trifluoroacetic acid) and allowed to dry. Ions were generated by irradiation with a pulsed nitrogen laser (337 nm) with a 4-ns pulse time. The instrument was operated in the delayed extraction mode with a 125-ns delay and an accelerating voltage of 20 kV. The grid voltage was 93.00%, guide wire voltage was 0.1%, the internal pressure was $<5 \times 10^{-7}$ torr (1 torr = 133 Pa), and the low mass gate was 875 Da. Spectra were generated from the sum of 100–200 laser pulses and acquired with a 500-MHz digitizer. $(Man)_5$-$(GlcNAc)_2$ oligosaccharide was used as an external molecular weight standard. All spectra were generated with the instrument in the positive-ion mode.

Mannosidase Assays. Fluorescence-labeled $(Man)_8$-$(GlcNAc)_2$ (0.5 μg) was added to 20 μl of supernatant and incubated for 30 h at room temperature. After incubation the sample was analyzed by HPLC with an Econosil NH_2 4.6 × 250-mm, 5-μm bead, amino-bound silica column (Alltech, Avondale, PA). The flow rate was 1.0 ml/min for 40 min, and the column was maintained at 30°C. After eluting isocratically (68% A:32% B) for 3 min, a linear solvent gradient (68% A:32% B to 40% A:60% B) was used over 27 min to elute the glycans (18). Solvent A was acetonitrile, and solvent B was an aqueous solution of ammonium formate, 50 mM (pH 4.5). The column was equilibrated with solvent (68% A:32% B) for 20 min between runs.

Results

Generation of an α-1,6-Mannosyltransferase Deletion Mutant in *P. pastoris*. In *S. cerevisiae* the *OCH1* gene product (Och1p) is an α-1,6-mannosyltransferase that initiates the outer-chain elongation of N-linked glycans in the early Golgi. An *och1* null mutant strain in *S. cerevisiae* shows no α-1,6-Man linkage to the core glycan structure and consequently lacks hyperglycosylation (19). To generate a deletion of the *OCH1* homolog in *P. pastoris* an *och1*::*URA3* mutant allele was constructed and transformed into a *ura3* strain of *P. pastoris* (JC308). Several *och1* mutant strains were identified and confirmed by PCR and Western blotting (Fig. 2*B*). The *P. pastoris och1* mutant strain exhibited temperature sensitivity and increased flocculation similar to that observed in *S. cerevisiae* (data not shown).

Glycan structures of heterologous proteins expressed in *P. pastoris* are heterogeneous, mostly consisting of $(Man)_{10-16}$-$(GlcNAc)_2$ mannans with varying degrees of charged glycans (10, 20). To monitor the effect of different engineering steps on the glycosylation *of P. pastoris*, a secreted form of K3 was used as a reporter protein. Hyperglycosylation observed by SDS/PAGE



analysis when K3 was expressed in a wild-type *P. pastoris* strain was eliminated in the *och1* mutant strain (Fig. 2*C*). N-glycans released from secreted K3 were analyzed directly by MALDI/TOF mass spectrometry. N-glycans of K3 expressed in a *P. pastoris* wild-type strain were found to have a molecular mass consistent with $(Man)_{10-16}$-$(GlcNAc)_2$, confirming previous findings in this yeast (Fig. 3*A*). By comparison, the N-glycans of K3 in an *och1* mutant strain revealed predominantly $(Man)_{8-12}$-$(GlcNAc)_2$, representing a noticeable shift to smaller glycans (Fig. 3*B*) and a complete elimination of the smearing observed in the SDS/PAGE analysis (Fig. 2*C*). To rule out the possibility of protein-specific interactions a second reporter protein, full-length human IFN-β, was used and revealed identical results (data not shown).

Construction of ER/Golgi leader, α-1,2-Mannosidase, and GnTI Libraries. To sequentially localize different mannosidases and GnTIs along the early secretory pathway of *P. pastoris* three separate gene libraries were designed. The first library (the leader library) contained DNA fragments encoding N-terminal peptides of known type II membrane proteins that either localize in the ER or Golgi of *S. cerevisiae* and *P. pastoris*. They include Gls1, Mns1, Sec12, Mnn9, Van1, Anp1, Hoc1, and Mnn10, Mnn11 from *S. cerevisiae* and Och1 and Sec12 from *P. pastoris*. The generation of the respective DNA constructs is exemplified by the construction of Mns1, Mnn9, and Mnn10 leaders in *Materials and Methods*. A second library contained catalytic domains of α-1,2-mannosidases from *Homo sapiens*, *Mus musculus*, *Aspergillus nidulans*, *C. elegans*, *Drosophila melanogaster*, and *Penicillium citrinium*. The generation of the respective DNA constructs is exemplified by the construction of mannosidase IB from *C. elegans* in *Materials and Methods*. Finally, a third library contained catalytic domains of GnTI genes from *H. sapiens*, *C. elegans*, *Xenopus laevis*, *and D. melanogaster*. All libraries were designed in a way that any combination of a leader construct and a catalytic domain created a gene encoding a chimeric fusion protein.

Each leader fragment was represented in three lengths. The short form encoded the N-terminal cytoplasmic tail and the transmembrane domain. The long form encoded additional residues containing the complete stem region up to the respective catalytic domain, which was determined by sequence homology to known catalytically active fragments of such enzymes. The medium form was an intermediate version containing approximately half of the stem region in addition to the sequence encoded in the short form. Catalytic domains were selected to cover a wide range of pH optima as determined from literature data (e.g., *P. citrinium* and *M. musculus*) and temperature optima by selecting domains from organisms that exist at different temperatures (e.g., *C. elegans* and *H. sapiens*). Each catalytic domain was represented in several lengths and generally lacked the native N-terminal cytosolic and transmembrane domain. Some of the catalytic domains were selected solely on the basis

Fig. 3. Positive-ion MALDI/TOF mass spectra of N-linked glycans released from K3. K3 was produced in *P. pastoris* strains BK64, BK64-1, YJN168, YJN188, and YJN201 and purified from culture supernatants by Ni-affinity chromatography. The glycans were released from K3 by peptide *N*-glycosidase F treatment. The released N-linked glycans were analyzed by MALDI/TOF mass spectrometry, typically appearing as the sodium or potassium adducts. (*A*) BK61, wild-type strain of *P. pastoris* expressing K3. (*B*) BK64-1, *och1* deletion expressing K3. (*C*) YJN168, *och1* deletion expressing K3, *K. lactis* UDP-GlcNAc transporter, and *C. elegans* α-1,2-mannosidase IB fused to *MNS1*. (*D*) YJN188, *och1* deletion expressing K3, *K. lactis* UDP-GlcNAc transporter, and *C. elegans* α-1,2-mannosidase IB fused to *MNN10*. (*E*) YJN201, *och1* deletion expressing K3, *K. lactis* UDP-GlcNAc transporter, *C. elegans* α-1,2-mannosidase IB fused to *MNS1*, and human GnTI fused to *MNN9*. (*F*) YJN201 after β-*N*-acetylhexosaminidase treatment. M, Man.

Table 1. Relative amount of $(Man)_5$ on secreted K3

Amount of $(Man)_5$ on secreted K3, % of total glycans	Number of constructs (%)
ND*	19 (3.1)
0–10	341 (56.1)
10–20	50 (8.2)
20–40	75 (12.3)
40–60	72 (11.8)
>60	51 (8.4)†
Total	608 (100)

Six hundred and eight different strains of *P. pastoris* (*och1*) were generated by transforming them with a single construct of a combinatorial genetic library that was generated by fusing 19 α-1,2-mannosidase catalytic domains to 32 fungal ER and cis-Golgi leaders.

*Several fusion constructs were not tested because the corresponding plasmids could not be propagated in *E. coli* before transformation into *P. pastoris*.

†Clones with the highest degree of $(Man)_5$ trimming (30/51) were analyzed further for mannosidase activity in the supernatant of the medium. The majority (28/30) displayed detectable mannosidase activity in the supernatant (e.g. Fig. 4*B*). Only two constructs displayed high $(Man)_5$ levels while lacking mannosidase activity in the medium (e.g. Fig. 4*C*).

of sequence homology to other known α-1,2-mannosidases (e.g. *C. elegans*) or GnTIs and had not been characterized previously.

After screening the fusion libraries chimeric constructs were identified that displayed a high degree of Man-trimming activity and UDP-GlcNAc transfer activity on the reporter protein K3. A detailed analysis of the characteristics of the fusion libraries will be published elsewhere (B.-K.C., P.B., R.C.D., S.R.H., A. Stadheim, H.L., R.G.M., J.H.N., S.W., and T.U.G., unpublished data).

Expression of α-1,2-Mannosidase Fusion Constructs in a *P. pastoris och1* Mutant Strain. After screening a library of 608 leader/α-1,2-mannosidase fusions targeted to the ER and early Golgi, several clones were identified that produced N-glycans consistent with a mass of $(Man)_5$-$(GlcNAc)_2$. Specifically, a putative *C. elegans* homolog of known α-1,2-mannosidases showed a high degree of trimming to $(Man)_5$-$(GlcNAc)_2$ when fused to both the *S. cerevisiae MNS1* and *MNN10* leader-encoding fragments. Although these constructs and several others resulted in glycans, which were 70–80% or more $(Man)_5$-$(GlcNAc)_2$ (Fig. 3 *C* and *D*), >56% of the fusions resulted in <10% $(Man)_5$-$(GlcNAc)_2$ (Table 1). These data clearly emphasize the importance of choosing the proper combination of (*i*) a localization sequence and (*ii*) an α-1,2-mannosidase catalytic domain of the proper length. Fungal α-1,2-mannosidases with acidic pH optima (e.g., *P. citrinium* and *A. nidulans*), when expressed as fusions with the leader library, generally resulted in low $(Man)_5$-$(GlcNAc)_2$ yields (data not shown) consistent with previous findings (11, 21).

Because previous researchers have found that trimming to $(Man)_5$-$(GlcNAc)_2$ was often accompanied by leakage of mannosidase into the medium (22), we further investigated whether Man trimming occurred *in vivo*, in the Golgi, or *ex vivo* after secretion of the protein into the medium. To determine the extent of mannosidase activity in the medium, 2-aminobenzamide-labeled $(Man)_8$-$(GlcNAc)_2$ was used to assay the culture supernatant. Many of the efficient $(Man)_5$-$(GlcNAc)_2$-producing constructs displayed a high degree of mannosidase activity in the supernatant, suggesting that at least some of the observed $(Man)_5$-$(GlcNAc)_2$ structures were produced *ex vivo*. However, by applying a double screen we were able to identify specific chimeric fusions that were entirely retained intracellularly while at the same time displaying high *in vivo* α-1,2-mannosidase activity (Fig. 4*C*).

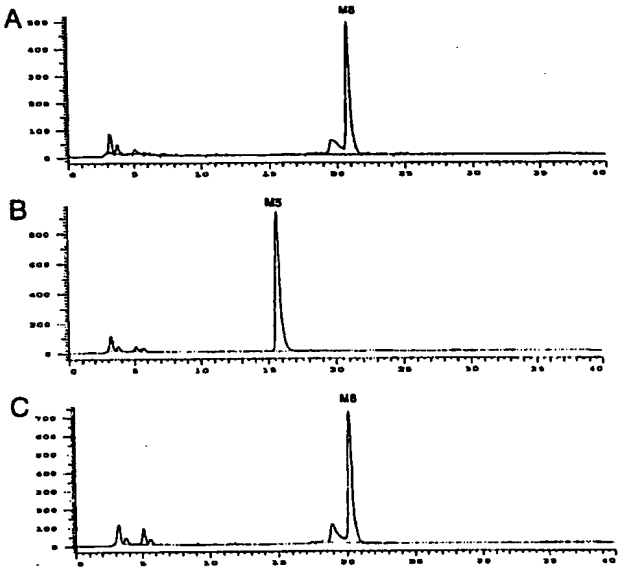


Fig. 4. Extracellular activity of *C. elegans* α-1,2-mannosidase IB. 2-Aminobenzamide-labeled $(Man)_8$-$(GlcNAc)_2$ was incubated with culture supernatants at 25°C for 30 h and analyzed by HPLC. (*A*) $(Man)_8$-$(GlcNAc)_2$ standard in BMMY medium (no cells). (*B*) Supernatant from YJN188. (*C*) Supernatant from YJN168. M, Man.

Expression of GnTI Fusion Constructs in a *P. pastoris och1* Mutant Strain Containing an Active α-1,2-Mannosidase. Further humanization of $(Man)_5$-$(GlcNAc)_2$ glycan structures involves the subsequent *in vivo* conversion of $(Man)_5$-$(GlcNAc)_2$ to GlcNAc-$(Man)_5$-$(GlcNAc)_2$. This step requires UDP-GlcNAc as a substrate and involves the transfer of GlcNAc to $(Man)_5$-$(GlcNAc)_2$ by GnTI (Fig. 1). To ensure sufficient levels of UDP-GlcNAc in the Golgi we cloned the UDP-GlcNAc transporter from *K. lactis* into an *och1* mutant strain, which also displays high *in vivo* mannosidase activity.

A leader/GnTI fusion library containing 67 constructs was screened. Among several active fusion constructs, one consisting of human GnTI and a leader sequence from *S. cerevisiae MNN9* was particularly active, and the corresponding strains yielded K3-containing glycans with a mass consistent with GlcNAc-$(Man)_5$-$(GlcNAc)_2$ almost exclusively (Fig. 3*E*). Moreover, these glycans were converted completely to $(Man)_5$-$(GlcNAc)_2$ by *in vitro N*-acetylhexosaminidase digestion, further indicating that this strain secretes protein almost uniformly modified with glycans of the structure GlcNAc-$(Man)_5$-$(GlcNAc)_2$, a well described human glycosylation intermediate (Fig. 3*F*). Importantly, GnTI activity was assayed in the culture medium of this strain and found to be absent (data not shown). Finally, to determine whether the UDP-GlcNAc transporter was required to provide sufficient amounts of substrate for GnTI, a strain producing $(Man)_5$-$(GlcNAc)_2$ was transformed with the same GnTI construct in the absence of the transporter. Although some GlcNAc-$(Man)_5$-$(GlcNAc)_2$ was observed from the purified K3, a large percentage of glycans consistent with $(Man)_5$-$(GlcNAc)_2$ remained, indicating that GlcNAc transfer was less efficient than in the strain containing the transporter (data not shown). This result confirms the purported necessity of a UDP-GlcNAc transporter for efficient GlcNAc transfer originally found in *K. lactis* (23).

Discussion

P. pastoris is one of several yeasts capable of high-level production of heterologous glycoproteins (24). As with any other fungal

protein-expression systems, the heterologous glycoprotein is glycosylated in a fungal-like fashion generally involving the addition of α-1,6- and α-1,2-linked Man and mannosylphosphate to the $(Man)_8$-$(GlcNAc)_2$ core (Fig. 1; ref. 25). This yeast-type glycosylation pattern is recognized by the human immune system, which renders the underlying protein unfit for therapeutic use. In view of the current shortage of efficient glycoprotein-expression systems we have focused our efforts on engineering commercially relevant yeast and filamentous fungi to produce N-glycans with human-like glycosylation structures. In this study we report the construction of a strain that produces high levels of a reporter protein modified with a human glycosylation intermediate in the *P. pastoris* system.

The first step in attaining complex human-type glycans in a fungal system is to eliminate yeast-type glycosylation. To accomplish this, the *P. pastoris OCH1* gene was deleted in a strain secreting the reporter protein K3. Yeast-type hyperglycosylation was abrogated in this strain as observed by silver staining of secreted K3 (Fig. 2*C*), and when released N-glycans were analyzed by MALDI/TOF, a general trend toward smaller structures was observed (Fig. 3*B*).

Once an appropriate core high-Man glycan is obtained, the next immediate step in the conversion to human-type N-glycans involves the functional expression and localization of an α-1,2-mannosidase. This enzyme will trim the $(Man)_8$-$(GlcNAc)_2$ core structure to $(Man)_5$-$(GlcNAc)_2$ and thereby generate the structure that is capable of receiving the GlcNAc that initiates the formation of hybrid N-glycans. A very similar approach was taken in a triple mutant *och1 mnn1 mnn4* strain of *S. cerevisiae* (11). ER localization of a fungal α-1,2-mannosidase (from *Aspergillus saitoi*) was accomplished by adding the tetrapeptide HDEL as an ER retrieval tag to the C terminus of the gene. By using the *S. cerevisiae* GAPDH promoter and a multicopy number plasmid, mannosidase activity was detectable in cell-free extracts; however, only 27% of the N-glycans of an endogenous marker protein (carboxypeptidase Y) were trimmed from $(Man)_8$-$(GlcNAc)_2$ to $(Man)_5$-$(GlcNAc)_2$ *in vivo*. Although not entirely successful, the pioneering work of Chiba *et al.* (11) demonstrates that N-glycans from *S. cerevisiae* can be modified substantially by engineering glycosylation pathways. Here the localization of catalytic domains to the secretory pathway of the related yeast *P. pastoris* using yeast type II membrane protein leader domains is demonstrated. The benefit of a large library of leader domains along with an equally diverse library of catalytic domains allowed for the selection of the most active fusion constructs from a pool of >600 candidates, many of which were marginally active or not at all. Despite the lack of success of many constructs, a few particularly active enzyme fusions were able to trim the core $(Man)_{8-12}$-$(GlcNAc)_2$ glycans observed in the *och1* mutant strain to $(Man)_5$-$(GlcNAc)_2$. Two of these constructs described here, which are fusions of a putative *C. elegans* α-1,2-mannosidase with different yeast type II leader domains, are able to do so at high efficiency (>75%).

The correct localization of enzymes involved in glycosylation is critical to allow for the sequential glycan modifications as glycoproteins proceed through the secretory pathway. However, one additional concern has come from another previous attempt to engineer glycans in *P. pastoris*, which was undertaken with an α-1,2-mannosidase from *T. reesei* (22). In this study immunofluorescent microscopy was used to demonstrate that a mannosidase-myc-HDEL fusion localized primarily in the ER of *P. pastoris*; however, leakage into the medium was also observed by Western blotting. Because the secreted glycoprotein of interest will be in the supernatant for many hours, the cosecretion of mannosidases into the medium is of concern because it can be misinterpreted as *in vivo* activity. It is important to generate $(Man)_5$-$(GlcNAc)_2$ structures *in vivo*, and early in the secretory pathway, if subsequent conversion to complex glycans is to be achieved. Although it is well established that the *ERD2*-based retrieval system is leaky and retention of the HDEL-tagged mannosidase in the secretory pathway cannot be ensured (26), the same concern is emphasized further by the striking difference demonstrated here between the two different *C. elegans* α-1,2-mannosidase IB fusion constructs. Thus, the use of a series of different fusion constructs with many differentially localized type II domains has allowed us to screen for chimera with activity that is completely *in vivo*.

After efficient trimming of the core glycan to $(Man)_5$-$(GlcNAc)_2$ the next step in the conversion of high Man-type glycans to hybrid- and complex-type glycans involves the expression and localization of the enzyme GnTI. Here again a library of GnTI catalytic domains was used and allowed for the screening for and selection of several particularly active GnTI fusion constructs for further study. One particularly active fusion using the human GnTI catalytic domain shown here converts the $(Man)_5$-$(GlcNAc)_2$ substrate to the desired GlcNAc-$(Man)_5$-$(GlcNAc)_2$ product almost quantitatively, an activity that was shown to be completely *in vivo*. Furthermore, the demonstrated activity of GnTI on the $(Man)_5$-$(GlcNAc)_2$ substrate is the best evidence that the α-1,2-mannosidase activity is indeed occurring in the secretory pathway. This is a demonstration of a high-level hybrid N-glycan modification of a secreted protein in yeast and represents a significant step toward the ability to express fully human glycoproteins in yeast. Although the generated structures are expected to be nonimmunogenic in humans, additional Man removal (i.e., the removal of 1,6- and 1,3-Man from the trimannose core) and further addition of β-1,2-GlcNAc will be required to generate complex N-glycans of therapeutic utility (e.g., for the production of monoclonal antibodies).

We thank Beata Bobrowicz, Teresa Mitchell, Sebastian Rausch, Andy Stadheim, Amelia Walling, and Harry Wischnewski for technical assistance. We gratefully acknowledge the gift of strains and reagents from Judah Folkman, James Cregg, Nick Menhart, Robert Barsteadt, Carlos Hirschberg, and Takashi Yoshida. We also thank Phil Robbins and Roger Bretthauer for their encouragement and continued support.

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EXHIBIT D

Production of Complex Human Glycoproteins in Yeast

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We report the humanization of the glycosylation pathway in the yeast *Pichia pastoris* to secrete a human glycoprotein with uniform complex N-glycosylation. The process involved eliminating endogenous yeast glycosylation pathways, while properly localizing five active eukaryotic proteins, including mannosidases I and II, *N*-acetylglucosaminyl transferases I and II, and uridine 5′-diphosphate (UDP)–*N*-acetylglucosamine transporter. Targeted localization of the enzymes enabled the generation of a synthetic in vivo glycosylation pathway, which produced the complex human *N*-glycan *N*-acetylglucosamine$_2$–mannose$_3$–*N*-acetylglucosamine$_2$ ($GlcNAc_2Man_3GlcNAc_2$). The ability to generate human glycoproteins with homogeneous *N*-glycan structures in a fungal host is a step toward producing therapeutic glycoproteins and could become a tool for elucidating the structure-function relation of glycoproteins.

The ability to produce recombinant human proteins has led to major advances in human health care and remains an active area of drug discovery. Some proteins such as insulin, which are not glycosylated in their native state, do not require glycosylation and can thus be expressed in prokaryotic hosts that lack a glycosylation machinery, such as *Escherichia coli*. Most therapeutic proteins, however, require the cotranslational addition of glycans to specific asparagine residues of the protein to ensure proper folding and subsequent stability in the human serum. For therapeutic use in humans, glycoproteins require human-like N-glycosylation. Mammalian cell lines that are able to replicate human-like glycoprotein processing have several drawbacks including low protein titers, long fermentation times, heterogeneous products, and ongoing viral containment issues.

Yeast and filamentous fungi are robust industrial fermentation organisms that can be grown to high cell density in chemically defined medium. However, glycoproteins derived from fungal expression systems contain nonhuman *N*-glycans of the high mannose type (Fig. 1), which are immunogenic in humans and thus of limited therapeutic value (*1*). Nevertheless, fungi and mammals share initial steps of protein N-glycosylation, which involve the following: (i) the site-specific transfer of $Glc_3Man_9GlcNAc_2$ from the luminal side of the endoplasmic reticulum (ER) to the de novo synthesized protein by an oligosaccharyltransferase complex, (ii) trimming by glucosidases I and II, and (iii) the removal of one specific terminal α-1,2-mannose residue by an ER-residing α-1,2-mannosidase. These sequential steps lead to the formation of $Man_8GlcNAc_2$ structures (isomer Man8B) (Fig. 1), the *N*-glycan found on most glycoproteins leaving the fungal or mammalian ER. After the export of predominantly $Man_8GlcNAc_2$-containing glycoproteins to the Golgi, *N*-glycan processing pathways diverge notably between mammals and yeast (*2*). The human Golgi contains several α-1,2-mannosidases (IA, IB, and IC), which remove mannose to yield $Man_5GlcNAc_2$, a precursor for complex *N*-glycan formation (Fig. 1).

In *Saccharomyces cerevisiae*, N-glycosylation involves the addition of numerous mannose sugars throughout the entire Golgi, which often leads to hypermannosylated *N*-glycans with more than 100 mannose residues. When this first α-1,6-mannose is added by 1,6-mannosyltransferase (Och1p), additional α-1,6-mannosyltransferases extend the α-1,6-chain, which then becomes the substrate for α-1,2- and α-1,3-mannosyltransferases, as well as phospho-mannosyltransferases that add yet more mannose sugars to the growing *N*-glycan structure (*3*).

Pichia pastoris is a methylotrophic yeast with a glycosylation machinery that is similar to that of *S. cerevisiae* and is frequently used for the expression of heterologous proteins (*4*). YJN201, a recombinant strain of *P. pastoris*, lacks endogenous Och1p activity, and contains three heterologous genes encoding α-1,2-mannosidase localized to the ER, UDP-GlcNAc transporter, and GlcNAc transferase I localized to the Golgi (Fig. 1) (*5*). Two of these proteins (MnsI and GnTI) are synthetic fusions between fungal type II membrane proteins and catalytic domains from *Caenorhabiditis elegans* and humans, respectively. YJN201 has the ability to secrete the kringle 3 domain of human plasminogen (K3) as a reporter protein, with *N*-glycans of the hybrid type (*5*). Here, we further humanize the N-glycosylation pathways in *P. pastoris*.

To identify a compatible combination of MnsII and GnTII, a two-stage approach was chosen. First, a combinatorial library of several MnsII catalytic domains was fused to a signal peptide library of more than 60 fungal type II membrane localization signals. The resulting 500 combinatorial fusion constructs were introduced into the *P. pastoris* strain YSH1, described in the subsequent paragraph, capable of producing the human precursor of complex glycosylation, $GlcNAcMan_5GlcNAc_2$ (Fig. 2B, compare Fig. 2A) on the reporter K3. Only a small subset of strains (<5%) were capable of quantitatively converting $GlcNAcMan_5GlcNAc_2$ to $GlcNAcMan_3GlcNAc_2$ (Fig. 2C). These strains were isolated and subsequently transformed with a combinatorial library of several hundred GnTII–leader peptide fusions. Screening for the presence of $GlcNAc_2Man_3GlcNAc_2$ allowed the isolation of strains that were able to secrete homogeneous complex glycan (Fig. 2D), as exemplified by strain YSH44.

Pichia pastoris YSH44 was engineered from BK64-1 (*6*), an *och1* deletion mutant secreting K3, a reporter protein with a single N-linked glycosylation site (*5*). After the introduction of the *Kluyveromyces lactis* uridine 5′-diphosphate (UDP)-GlcNAc transporter (*5*), the strain was further used for the introduction and screening for active α-1,2-mannosidase fusions. Among 608 chimera, the mouse MnsIA catalytic domain fused to the N-terminal localization peptide of the ER protein Sec12 from *S. cerevisiae* was selected because of its ability to produce primarily $Man_5GlcNAc_2$. Subsequent introduction of a human GnTI fusion library allowed the isolation of a strain (PBP-3) able to produce primarily $GlcNAcMan_5GlcNAc_2$ structures (Fig. 2B). The URA marker in PBP-3 was recovered, which produced the strain YSH1. Transformation of the latter strain with a library of MnsII catalytic domains fused to a library of localization peptides permitted the isolation of yeast strains producing $GlcNAcMan_3GlcNAc_2$ as the primary *N*-glycan, with some $GlcNAcMan_4GlcNAc_2$ and $GlcNAcMan_5GlcNAc_2$ also present (Fig. 2C). This strain, YSH37, contains a catalytic domain of the *Drosophila melanogaster* mannosidase II (*7*), fused to the N-terminal localization signal of the *S. cerevisiae* Golgi protein Mnn2. Finally, we generated YSH44 by introducing a GnTII–localization peptide library and screening for strains that are able to

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produce K3 with uniform $GlcNAc_2Man_3GlcNAc_2$ structures (Fig. 2D). Specifically, this strain contained the catalytic domain of GnTII from *Rattus norvegicus* and the leader peptide from *S. cerevisiae* protein Mnn2. As expected, the *N*-glycans from YSH44 were substrates for both hexosaminidase and β-1,4-galactosyltransferase (Fig. 2, E and F). Unlike the parent strain BK64-1, YSH44 no longer displayed flocculation behavior in culture and was able to produce similar amounts of recombinant protein when compared with the commercially available wild-type *P. pastoris* strain GS115 (Fig. 3). These strains both demonstrated a similar degree of occupancy on the single N-linked site of K3 (~100%) (Fig. 3).

Being able to produce *N*-glycans of high purity in vivo depends on the efficiency of individual processing steps throughout the secretory pathway and the absence of local competing reactions for the same substrate. For example, YSH37 shows the consequence of producing a glycan intermediate in vivo that can also serve as a substrate for competing pathways. Although $GlcNAcMan_3GlcNAc_2$ represented the main *N*-glycan structure produced by this strain, we found two additional structures with masses corresponding to $GlcNAcMan_4GlcNAc_2$ and $GlcNAcMan_5GlcNAc_2$. We performed detailed analyses by mass spectrometry, high-performance liquid chromatography, and enzymatic digestions to determine the structure of these contaminating glycans. The fourth and fifth mannose residues on the $GlcNAcMan_3GlcNAc_2$ core were sensitive to α-1,2-mannosidase digest, which led us to conclude that mannosidase II conversion was complete and that subsequent α-1,2-mannosyltransferase activity was involved.

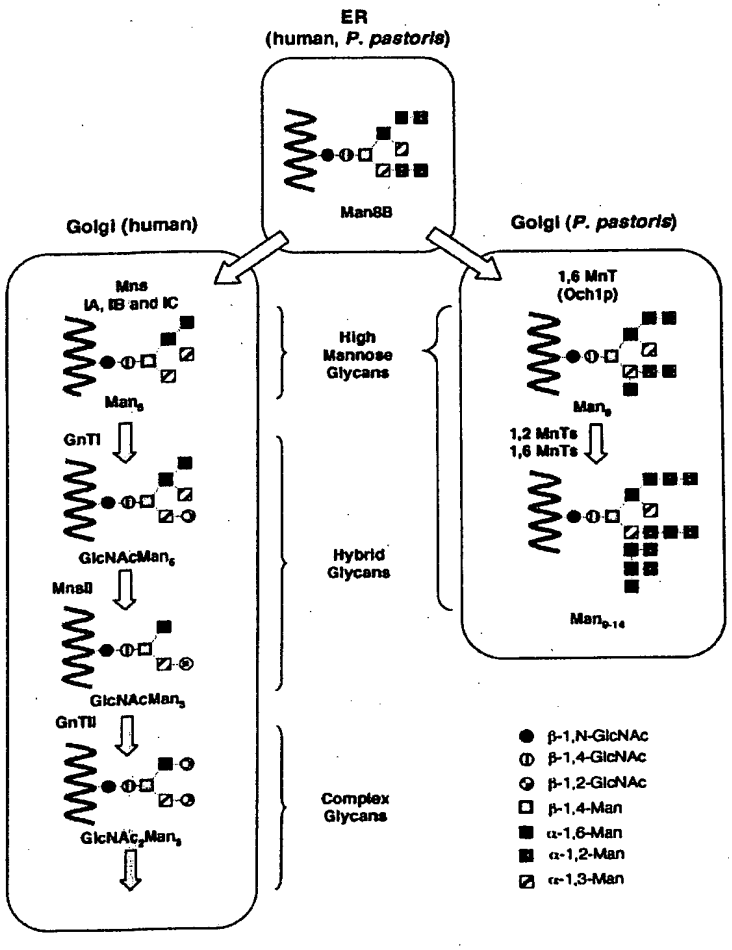

Fig. 1. N-linked glycosylation pathway in humans and in *P. pastoris*. Mns, α-1,2-mannosidase; MnsII, mannosidase II; GnT1, β-1,2-*N*-acetylglucosaminyltransferase I; GnTII,β-1,2-*N*-acetylglucosaminyltransferase II; MnT, mannosyltransferase.

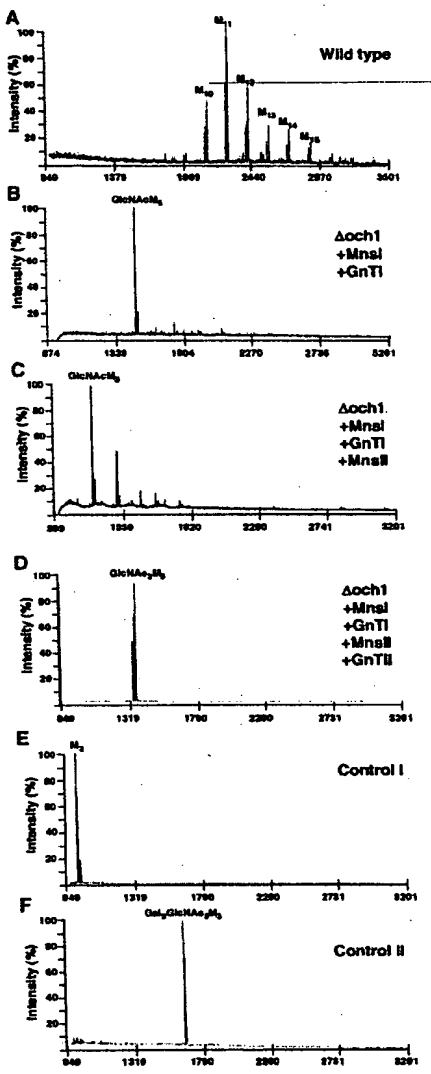

Fig. 2. Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF) spectra of N-linked glycans released from recombinant K3. K3 was produced in *P. pastoris* strains BK64 (5) [GS115 wild-type control (Invitrogen, Carlsbad, USA)], PBP-3, YSH37, and YSH44 and purified from culture supernatants by Ni-affinity chromatography. The glycans were released from K3 by peptide–*N*-glycosidase F treatment. The released N-linked glycans were analyzed by MALDI-TOF mass spectrometry (positive mode), typically appearing as the sodium or potassium adducts. (A) BK64, (B) PBP-3, (C) YSH37, (D) YSH44, (E) glycans from YSH44 after β-*N*-acetylhexosaminidase treatment, and (F) glycans from YSH44 after β-1,4-galactosyltransferase treatment. To simplify labeling of glycoforms, the two core GlcNAc residues, although present in all species, have been omitted. M, mannose.

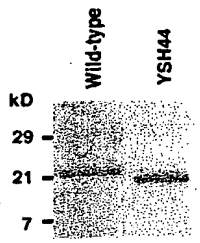

Fig. 3. Comparative expression levels of K3 in wild-type (BK64) (5) versus YSH44 (6) *P. pastoris*. Analysis of the reporter protein K3 by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (6).

This was further substantiated by the introduction of GnTII, which preempted the action of α-1,2-mannosyltransferase and led to the formation of uniform $GlcNAc_2Man_3GlcNAc_2$ structures (Fig. 2, D and E).

In the glycosylation process, multiple enzymes compete for the same transient glycan structures, which typically leads to heterogeneous mixtures of glycoforms. Here the fungal glycosylation pathway was reengineered to mirror the processing of human *N*-glycan structures. However, unlike the human pathway, which typically results in an array of glycoforms, this system yielded essentially homogeneous glycoforms.

To better understand the relation between N-glycosylation and protein function, generating uniform glycoproteins with specific *N*-glycan structures will prove to be of great utility (*8*). At present, the interpretation of these relations is compromised by the heterogeneity of the glycoform pools that can be generated from mammalian sources. These pools are typically created by expressing glycoproteins in specific glycosylation mutants or by ex vivo enrichment, such as lectin chromatography or enzymatic treatment. Moreover, even when a particular structure is identified, it is difficult to produce these structures at a commercial scale. Being able to engineer human glycosylation pathways into yeast strains able to express proteins with uniform *N*-glycan structures offers a more practical solution to this problem. When single proteins from a library of genetically engineered yeasts can be expressed and when each produces a defined and uniform glycoform, glycoprotein libraries can be generated to elucidate specific structure-function relations and to identify the most efficacious glycoform for a particular biological function. Once identified, a particular glycoform can be readily produced at industrial scale because of the rapid and well-established scale-up of yeast fermentations.

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Supporting Online Material
www.sciencemag.org/cgi/content/full/301/5637/1244/DC1
Materials and Methods
References

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Representation of Action Sequence Boundaries by Macaque Prefrontal Cortical Neurons

Naotaka Fujii and Ann M. Graybiel*

Complex biological systems such as human language and the genetic code are characterized by explicit markers at the beginning and end of functional sequences. We report here that macaque prefrontal cortical neurons exhibit phasic peaks of spike activity that occur at the beginning and endpoint of sequential oculomotor saccade performance and have the properties of dynamic start- and end-state encoders accompanying responses to sequential actions. Sequence bounding may thus reflect a general mechanism for encoding biological information.

Speaking, hitting a fastball, and playing the flute are premier examples of behaviors requiring meticulous sequencing of actions. These are learned skills, but the ability to carry out sequences of movements is fundamental to most normal behaviors of humans and other animals (*1*). These sequential behaviors extend over time, so that temporal order as well as spatial order must be regulated. Many levels of the neuraxis have been implicated in the control of sequential behaviors (*2–6*). The prefrontal cortex, however, is a key region involved in the planning and automatization of the sequential behaviors that enable us to produce a smooth flow of actions or thoughts across time (*7–11*).

Implicit in the structure of such sequences is that they have a clear beginning and end, and explicit designations of start and end states have proven useful in models of sequential behavior (*12*). To test for such representations of start and end states, we recorded from multiple neurons in the prefrontal cortex and, for comparison, in the frontal eye field (FEF), as highly trained macaque monkeys performed up to seven sequential saccades (eye movements to redirect the line of sight) in blocks of trials (*13*) (Fig. 1A). Successive trials had a clear beginning (the illumination of a central red fixation point), a clear movement period (in which the monkey made saccades to successively presented red visual targets), and a final reward delivery period (in which the monkeys received juice or water for correct performance). In the standard task, different sequences were presented in pseudo-random order within trial blocks. We analyzed neuronal spike activity in relation to successive task events throughout the trials and during the intertrial intervals (*13*).

Neurons in the FEF responded selectively to saccades made in particular directions (*14, 15*), and some showed sequence selectivity. Many neurons in the prefrontal cortex exhibited similar properties, but in addition they responded to multiple phases of the task and often exhibited tonic firing during the entire trial or during the movement period (*13, 16*). Figure 1B illustrates typical prefrontal responses during the four-saccade task, with peaks of activity for each saccade during the movement period and a smaller peak after initiating fixation.

Nearly half of the task-related prefrontal neurons (295 of 658), but only rare FEF neurons (13 of 116), had an additional phasic peak in firing 270 to 280 ms after the sequence of saccades was completed. This "extra" peak occurred no matter how many saccades were made during the previous movement period (Fig. 1, B, E, and F) (fig. S1) (*13*). Even for a single saccade, there

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EXHIBIT E

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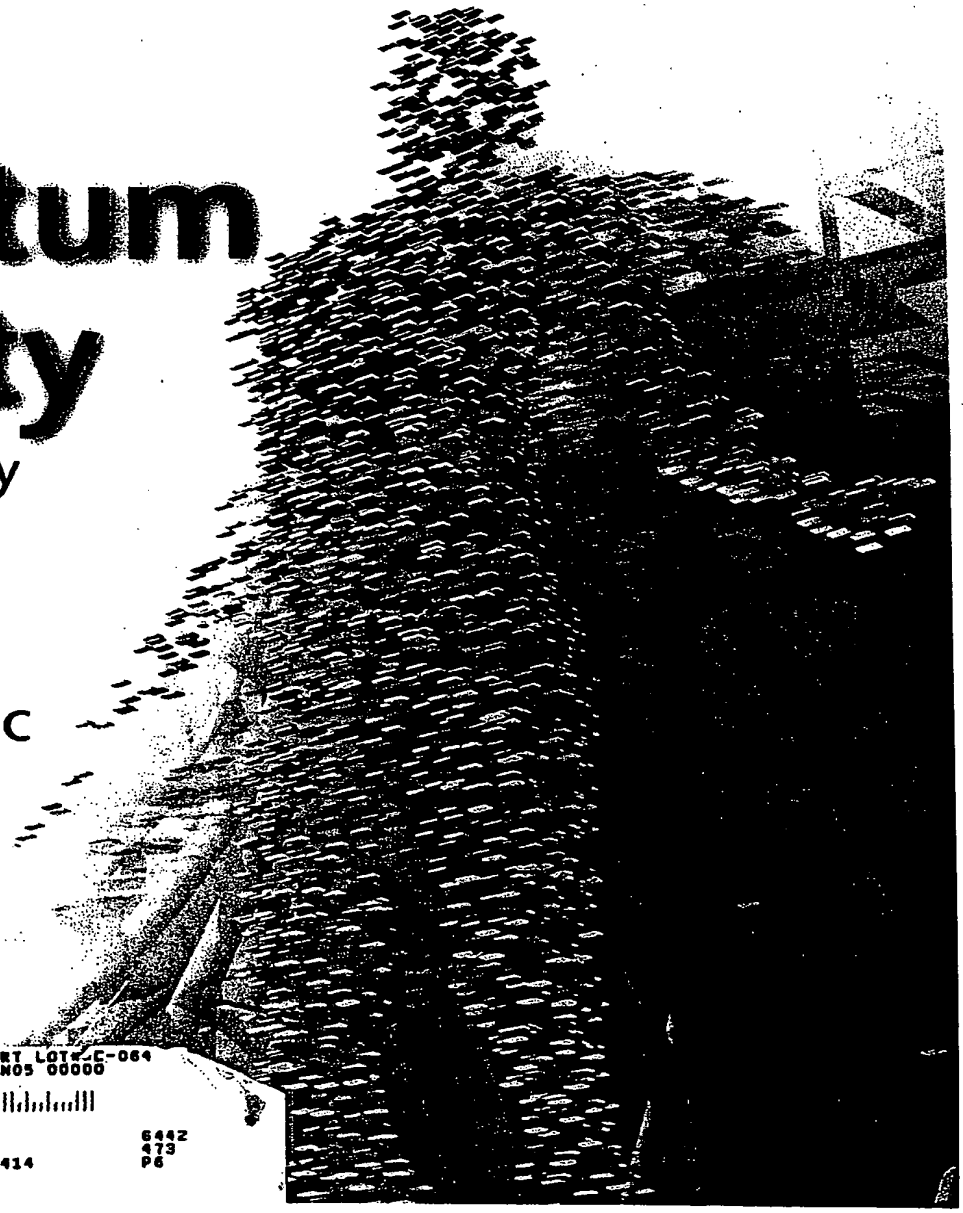


THE CANNIBALISTIC MILKY WAY

SCHIZOPHRENIA

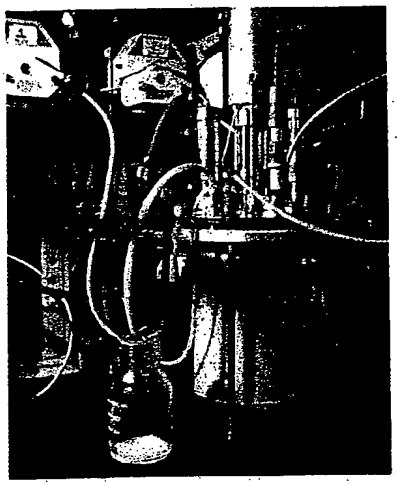
THE RISKS OF AN EARLIER SPRING

Supercharging Protein Manufacture

A career deviation leads to a dynamic approach to producing biotech drugs By GARY STIX

Tillman U. Gerngross came to Dartmouth College in the late 1990s as a tenure-track professor who wanted to study "green" plastics derived from plant-derived sugars. His first major project centered on performing an analysis of the costs and benefits of these supposed materials of the future.

In 1999 he published a paper in *Nature Biotechnology* that detailed the results of a life-cycle analysis of bioplastics manufacturing. It showed that making these purportedly eco-friendly products required more fossil fuels than fabricating petroleum-based plastics. "We have spent literally hundreds of millions of dollars developing these technologies to make green polymers. And at the end of the day, the net impact is going to be marginal," Gerngross says [see "How Green Are Green Plastics?" by Tillman U. Gerngross and Steven C. Slater; SCIENTIFIC AMERICAN, August 2000].



FERMENTER: A GlycoFi vessel is where protein-based drugs will be made in yeast.

The 1999 paper got a lot of attention. But it also spurred the Austrian native's decision to look for another line of research. "In one publication, I essentially divested myself of that [green plastics] work," he says. For a while, Gerngross became what he describes as a "poster boy for the debunking movement." But more scientist than pundit, he realized that he could not spend decades belaboring this one idea. The experience taught him that before taking on any new research endeavor, he should examine whether the scientific problem he had chosen to go after was really worth solving.

At the time, the Human Genome Project was entering its final stages. Trained as a chemical engineer and molecular biologist, Gerngross started to take a close look at all the steps leading from gene identification to the coding and making of proteins—the staple therapeutics in biotechnology. In particular, the manufacture of proteins caught his eye. "I realized that this is fairly medieval. It is a lengthy process that we can't control well, that has all sorts of shortcomings and that there ought to be a better way of making proteins." Today's standard method involves inserting a gene into Chinese hamster ovary cells or other mammalian cells, which then express the human protein; it can take two to three weeks to produce relatively small amounts of a protein-based drug.

Gerngross wondered whether generating human proteins in yeast might produce better results: "Yeast can make boatloads of protein, but they can't put the right sugars on the protein." Among other things, the sugars ensure that the protein folds properly and that it is thermodynamically stable. As he talked to colleagues, Gerngross realized that devising a production process for glycosylated proteins—ones with the desired sugars added—would meet his criterion for pursuing worthwhile research. "People said to me, 'This is a hard problem, but if you solve it, this would be a big deal.'"

Typically an academic applies for a government grant and sets to work with a few graduate students. As a newcomer to the field of glycobiology, Gerngross knew he had little chance of getting support through traditional funding routes—and if he did receive the money, it would take years to achieve substantive results: "By that time, the boat would have left and someone else would have picked this up."

Charles E. Hutchinson, a teaching partner and former dean of the engineering school at Dartmouth, was intrigued. He told Gerngross that the only way to proceed quickly would be to launch a company. A veteran of multiple start-ups, Hutchinson helped to interest a

venture-capital firm, Polaris Ventures in Waltham, Mass., in providing $600,000 in 2000. In exchange for an equity stake in the newly formed GlycoFi (short for "glycosylation fidelity"), the university agreed to let the two men use Gerngross's laboratory space on campus to get started. With a call extending a job offer to Stefan Wildt, a former postdoctoral colleague from the Massachusetts Institute of Technology, the company had become more than what Gerngross calls "a postal box and a cute idea."

Gerngross and his colleagues set about reengineering the glycosylation pathway of several yeasts, initially focusing on *Pichia pastoris,* which is widely used in the production of industrial enzymes. First, it was necessary to knock out the genes in yeast that encoded enzymes that would place the wrong sugars on a human protein, making it an immediate target for disposal by immune cells. Deleting genes was by far the simplest task.

The biggest challenge, and one that had foiled other investigators, came next: to create an assembly line of enzymes needed to put the appropriate sugars on a human protein being manufactured in the yeast cell. Kirin Brewery, for one, had inserted the human gene for a critical glycosylation enzyme in yeast, but little had happened. The GlycoFi team reasoned that for the enzyme to work, it would have to get to the right place in the yeast cell. The researchers attached a peptide, a small chain of amino acids, to the enzyme. This peptide zip code then directed the enzyme to either the yeast cell's endoplasmic reticulum or its Golgi apparatus.

In addition to helping the enzymes find their way in the cell, GlycoFi began a cross-species search to locate the best enzymes to perform the diverse reactions required to sugarcoat the human proteins. The enzymes were not always culled from human cells; rat, worm, plant or yeast enzymes sometimes carried out the reactions needed to glycosylate a human protein better than their human counterparts did. The genes for the best enzymes, whether rat or human, were engineered to express the correct peptide zip codes and then inserted into the yeast.

This sugar assembly line has functioned better than anyone expected. For reasons no one yet fully understands, the yeast does not appear to be weakened by this fiddling with its internal workings. The most recent report on GlycoFi's research—announcing the first production of a human protein decorated with complex sugar molecules—was published in the August 29, 2003, *Science.*

More still needs to be done before GlycoFi can offer a complete industrial platform that will compete with Chinese hamster ovary cells. The yeast must be engineered further to add the sugar sialic acid to a protein. But the possibility of making human proteins in yeast cells looms as a formidable technology. Gerngross notes that fermentation times in yeast may take three days, compared with two to three weeks in hamster cells. And both the amount of protein produced and the uniformity of the product show the promise of the technology. Lowered production costs from these improvements in manufacturing could potentially bring down the cost of biotechnology drugs.

"We hope to be able to produce longer-lasting and better drugs," Gerngross says. "You may not have to administer as much as you would with another drug to get the same therapeutic effect." GlycoFi might also make drugs that simply cannot be produced in mammalian cells. Gerngross points out that yeast, for example, can manufacture high concentrations of the properly glycosylated form of the protein alpha-1 antitrypsin, a deficiency of which can cause liver and lung disease. Creating the protein in hamster cells is impractical because of low yields.

The challenge that had foiled others was to create an assembly line of enzymes that put the appropriate sugars on a human protein made in yeast.

GlycoFi, now with 37 employees, has grown beyond the confines of Gerngross's college lab. Its new headquarters in Lebanon, N.H., was a presidential campaign stop for Senator Joseph Lieberman of Connecticut last July. The company is now closing its third round of venture financing, having brought in nearly $18 million since its inception. Moreover, it has already received some revenue from drugmakers such as Biogen Idec and Baxter Healthcare, which have each supplied a gene; in return, GlycoFi is providing the specified protein. So far, discerning how to put sugars on human proteins made in yeast looks like a problem well worth solving. ■

GLYCOFI (glycosylated protein)

EXHIBIT F

Research Focus

Genetic engineering of *Pichia pastoris* to humanize *N*-glycosylation of proteins

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The yeast *Pichia pastoris* is used extensively as the host cell for large-scale production of secreted recombinant proteins. Many proteins of pharmaceutical importance are *N*-glycosylated, and therefore require an expression host that yields *N*-linked oligosaccharides that are structurally and functionally identical to the human counterpart. The recent report by Choi *et al.* describes the use of combinatorial genetic libraries to alter the *N*-glycosylation pathway in *P. pastoris* to yield *N*-linked oligosaccharides with hybrid structures that are the same as the intermediates of mammalian-protein *N*-glycosylation. In view of recent progress in this area, the production of complex human glycans in yeasts is anticipated.

The advent of recombinant DNA technology has drastically improved the availability of therapeutic proteins, some of which were previously not obtainable in sufficient amounts from biological sources. Two protein expression systems, prokaryotic-cell and mammalian-cell cultures, have evolved as the primary hosts for the expression of biologically active recombinant proteins. More recently, the development of eukaryotic yeast expression-systems has become of great interest and importance. A crucial factor is that the proteins expressed must be glycosylated with saccharide structures that are native (or at least not antigenic) to humans. The recent publication by Choi *et al.* [1] details a significant step towards achieving this goal by reporting the first synthesis of a hybrid *N*-linked oligosaccharide in a yeast expression host.

Characteristics of the *Pichia pastoris* expression system

The use of *Pichia pastoris* for expression of foreign proteins offers several advantages, including (i) high titers of expressed proteins; (ii) relatively inexpensive growth media; (iii) the ability to *N*-glycosylate the expressed proteins; and (iv) the lack of α1,3-mannosyltransferase activity in the Golgi apparatus. Although the ability to glycosylate is essential for many therapeutically important proteins, it is also a serious disadvantage of yeast expression-systems because *N*-glycosylation in yeast usually involves glycans of the high mannose type with up to 100 or more mannose residues (hyperglycosylation) [2–4]. Extensive genetic alterations of the glycosylation pathway in *Pichia* (or other yeast strains) will therefore be required to overcome the aberrant non-human nature of yeast protein-glycosylation.

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N-glycosylation of proteins in yeast and mammals

The biosynthetic pathway leading to the formation of the protein *N*-linked $Man_8GlcNAc_2$ (isomer B) oligosaccharide involves several processing steps that are highly conserved in mammals and yeast. However, after formation of the Man_8GlcNA_2 intermediate, the two pathways diverge (Figure 1). Humans remove mannose residues, whereas yeasts and other fungi add mannose residues. Therefore to 'humanize' glycosylation in yeasts it is necessary to eliminate the transfer of an α1,6-linked mannose residue to prevent the formation of the $Man_9GlcNAc_2$ oligosaccharide precursor, which is known to be a substrate for hypermannosylation. Several basic genetic alterations are required to achieve this goal. Firstly, to prevent the addition of the α1,6-linked mannose residue to the α1,3-linked mannose-arm residue of the $Man_8GlcNAc_2$ oligosaccharide in the Golgi, the *OCH1* gene must be eliminated or inactivated. Secondly, a gene for α1,2-mannosidase activity (encoding mannosidase I-type) must be introduced to remove the three α1,2-linked mannose residues from the $Man_8GlcNAc_2$ oligosaccharide to form $Man_5GlcNAc_2$. Thirdly, a gene (encoding GlcNAc transferase I) for adding a β1,2-linked GlcNAc residue to the α1,3-linked mannose-arm residue must be added, resulting in a protein-linked oligosaccharide that is a common intermediate structure of mammalian glycoprotein synthesis. Finally, to generate complex-type oligosaccharide structures, the two remaining terminal α1,3 and α1,6 mannose residues linked to the α1,6-linked mannose-arm residue would have to be removed by introduction of another α-mannosidase gene (encoding mannosidase II-type), followed by a second GlcNAc transferase gene (encoding GlcNAc transferase II) that adds a β1,2-linked GlcNAc residue to the now-exposed α1,6-linked mannose-arm residue. Successful completion of these genetic-engineering steps would allow the generation of $GlcNAc_2Man_3GlcNAc_2$–protein-linked complex-type oligosaccharides that are the precursors for further addition of galactose and sialic acid residues.

Genetic alterations of the yeast *N*-glycosylation pathway

The feasibility of carrying out at least some genetic alterations in yeast while maintaining a viable cell has been demonstrated by several research groups. Chiba *et al.* [5] used *Saccharomyces cerevisiae* for the first reported synthesis of $Man_5GlcNAc_2$–protein. The host yeast strain, a triple mutant with dysfunctional *OCH1*, *MNN1* and *MNN4* genes (encoding the chain-initiating α1,6-mannosyltransferase, the chain-terminating

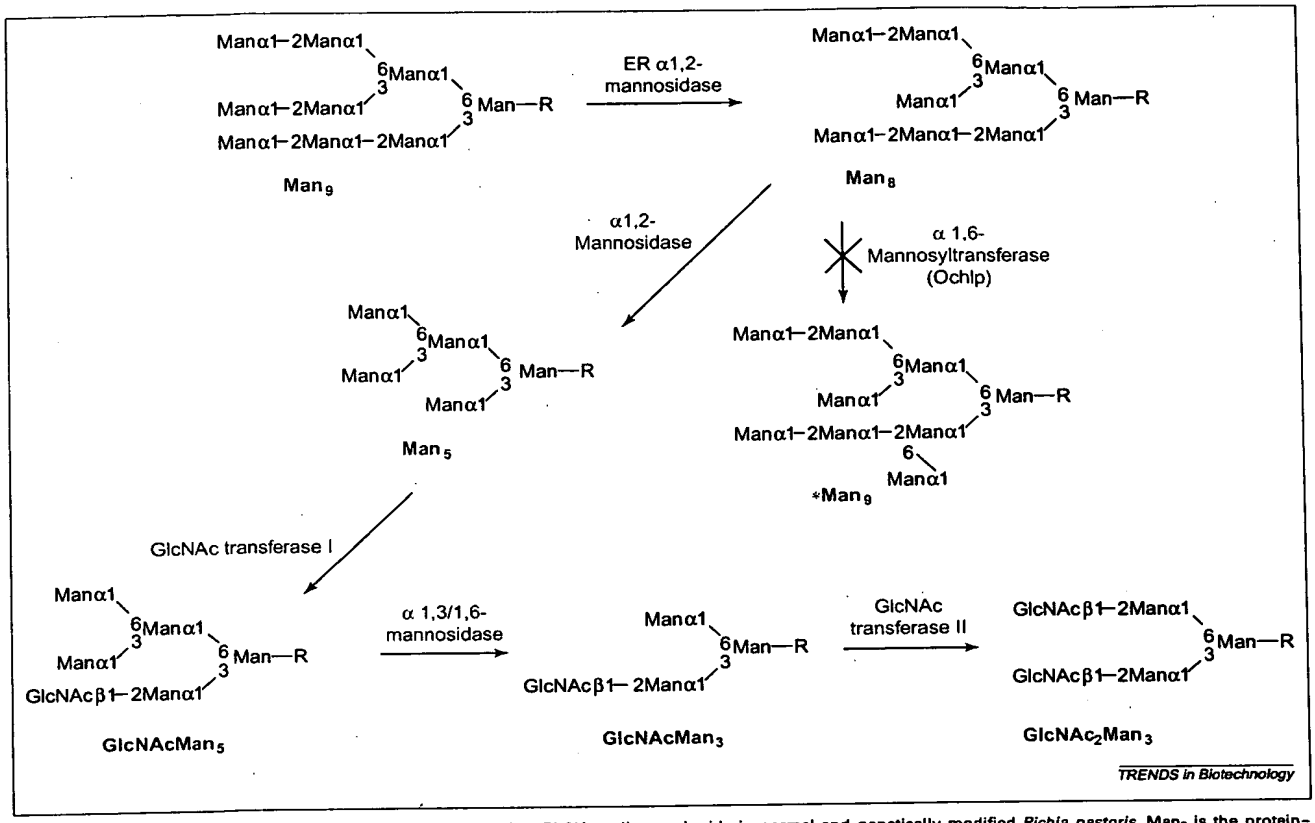

Figure 1. Alternate pathways for processing protein-*N*-linked $Man_9GlcNAc_2$ oligosaccharide in normal and genetically modified *Pichia pastoris*. Man_9 is the protein-*N*-linked oligosaccharide initially derived from $Glc_3Man_9GlcNAc_2$-pyrophosphorylidolichol in the endoplasmic reticulum (ER). Man_8 is a product of a specific α1,2-mannosidase in the ER of normal and genetically modified cells. Deletion of the protein encoded by *OCH1* (α1,6-mannosyltransferase) prevents the conversion of Man_8 to the specific $^*Man_9$ that would normally be further elongated and branched by Golgi α1,6- and α1,2-mannosyltransferases to form the hypermannosylated mannan. Further modification of the *OCH1*-negative cells by addition of another α1,2-mannosidase allows formation of the Man_5, which serves as a substrate for the added GlcNAc transferase I to yield the hybrid $GlcNAcMan_5$ protein-linked oligosaccharide. To generate the complex $GlcNAc_2Man_3$ precursor that would allow further addition of galactose and sialic acid residues requires the addition of an α1,3/1,6-mannosidase and a GlcNAc transferase II. Abbreviations: Asn, asparagine; ER, endoplasmic reticulum; GlcNAc, *N*-acetylglucosamine; Man, mannose; R, β1-4GlcNAcβ1-4GlcNAcβ1-*N*-AsnProtein.

α-1,3-mannosyltransferase, and a protein involved in phosphorylmannose transfer, respectively) synthesized $Man_8GlcNAc_2$-type proteins as the primary glycoform. After introduction of an *Aspergillus* α1,2-mannosidase gene (*msdS*) fused to an endoplasmic reticulum (ER) retention signal sequence (HDEL), cellular carboxypeptidase Y and cell-wall mannoproteins contained up to 27% and 10% $Man_5GlcNAc_2$ oligosaccharide, respectively, indicating that it can trim mannose residues *in vivo*, albeit at low efficiency. An important feature of this work was the requirement for a proper localization signal for the α1,2-mannosidase in the ER (resulting from the HDEL signal). The same authors noted that a chimeric α1,2-mannosidase fused to the Och1p transmembrane domain did not result in synthesis of $Man_5GlcNAc_2$–proteins in the recombinant yeast, and was mislocated in the ER, Golgi and/or cytoplasm. A similar approach was pursued in *P. pastoris* by Martinet and co-workers [6]. However, contrary to previous studies in *S. cerevisiae*, the goal of obtaining *N*-glycosylation mutants by $[H^3]$–mannose suicide selection [7] and by vanadate resistance [8] remained elusive. The authors found that introducing the catalytic domain of an α1,2-mannosidase from *Trichoderma reesei*, localized to the ER by a type II membrane domain, appeared to increase the degree of mannosylation, rather than decreasing it, as had been expected.

In subsequent work, the same group, following the approach used by Chiba *et al.*, partially localized the α1,2-mannosidase from *T. reesei* using an HDEL retrieval tag [9]. Localization to the ER was confirmed by fluorescent microscopy, but α1,2-mannosidase was also found in the medium, possibly because of over saturation of the HDEL retrieval mechanism. The effects of introduction of the α1,2-mannosidase on *N*-linked oligosaccharide processing were evaluated by co-expressing two secreted recombinant glycoproteins. The two proteins, *Trypanosoma cruzi trans*-sialidase or influenza virus haemagglutinin, were expressed as fusion proteins with the *S. cerevisiae* α-mating factor. Expression of the *trans*-sialidase under the control of the AOX1 promoter in the absence of co-expressed mannosidase yielded the *trans*-sialidase-containing $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ oligosaccharides. These oligosaccharides were mostly converted to $Man_5GlcNAc_2$ following *in vitro* digestions with purified α1,2-mannosidase, indicating the absence of terminal

1,6-linked mannose residues typically found in *P. pastoris*. Co-expression of the *trans*-sialidase with the *Trichoderma reesei* mannosidase, under the control of the GAP promoter, gave a secreted *trans*-sialidase that contained predominantly $Man_5GlcNAc_2$. Although trimming from $Man_8GlcNAc_2$ to the mammalian-type $Man_5GlcNAc_2$ oligosaccharide was observed, it was not conclusively shown exactly where mannosidase trimming occurred. Because mannosidase was found in the ER and in the medium (by fluorescence microscopy and Western blot, respectively), it remains unclear to what extent mannosidase activity occurred *in vivo*. Protein synthesis and secretion has been estimated to take ~15 minutes, leaving only a few minutes for the ER-localized mannosidase to act. However, once in the medium, the secreted *trans*-sialidase would be in the presence of co-secreted mannosidase for several hours. In addition, because the early Golgi α1,6-mannosyltransferase was not acting on this particular co-expressed glycoprotein, the conclusions are not representative of other co-expressed glycoproteins from this strain as shown by the more-typical wild-type glycosylation pattern of co-expressed haemagglutinin. The major oligosaccharides on the expressed haemagglutinin were $Man_{9-11}GlcNAc_2$, which are structural derivatives of the $Man_8GlcNAc_2$, containing 1–3 additional mannose residues added by the *OCH1*-encoded protein and perhaps other 1,6-mannosytransferases. These 1,6-linked mannose residues are recalcitrant to α1,2-mannosidase activity and consequently no $Man_5GlcNAc_2$ oligosaccharides could be obtained following the introduction of the ER-targeted *T. reesei* mannosidase. These data strongly suggest that the initiating α1,6-mannosyltransferase activity (Och1p) has to be eliminated before the introduction of α1,2-mannosidase activity.

Mannosylphosphorylation has been noted in several expressed recombinant proteins from *P. pastoris* [10,11]. This might alter structural or functional features of the glycoprotein and must be considered in genetic engineering of *P. pastoris*. The *MNN4* and *MNN6* genes in *S. cerevisiae* are involved in mannosylphosphorylation, and characterization of similar genes in *P. pastoris* could lead to a means of avoiding this type of oligosaccharide modification.

Use of combinatorial genetic libraries to humanize *N*-glycosylation in *P. pastoris*

The recent report by Choi *et al.* takes into consideration many of the cellular events described here and represents a major step in the 'humanization' of *N*-linked oligosaccharide pathways in fungal hosts. The authors focused on five major areas: (i) elimination of initiating α1,6-mannosyltransferase activity; (ii) proper subcellular localization of processing enzymes using combinatorial genetic libraries; (iii) using catalytic domains from several different sources, (iv) assuring the availability of UDP–GlcNAc in the Golgi; and (v) rapid screening of large numbers of recombinant glycoproteins for the desired oligosaccharide structure. By first deleting the *OCH1* gene (*S. cerevisiae* homolog), the authors prevented the formation of undesirable high-mannose structures that subsequently cannot be trimmed *in vivo* by α1,2-mannosidases. The *OCH1*-negative strain was used for transformation with genetic libraries that encoded synthetic α1,2-mannosidases to screen for the conversion of the $Man_8GlcNAc_2$–protein to $Man_5GlcNAc_2$–protein. All genetic libraries were constructed by combining *N*-terminal signal sequences of various type II ER or Golgi membrane proteins (mostly of fungal origin) with catalytic-domain sequences of α1,2-mannosidases, yielding an array of chimeric fusion-proteins. Selection of strains containing an active mannosidase fusion was accomplished by purifying a secreted reporter protein [kringle 3 domain (K3) of human plasminogen] from each strain grown in a 96-well plate, and monitoring the structures of the oligosaccharides released by protein *N*-glycanase (PNGase) using MALDI-TOF (matrix-assisted laser/desorption ionization time-of-flight) mass spectrometry. Several transformed strains (from >600) were identified that had acquired the ability to synthesize $Man_5GlcNAc_2$–K3 with high yield. In view of prior work that showed the co-localization of mannosidase inside and outside the cell, particular attention was paid to the localization of the mannosidase fusions and their respective site of action. Intracellular retention of mannosidase activity was demonstrated for at least one mannosidase construct by showing the absence of mannosidase activity in the medium while demonstrating high conversion to $Man_5GlcNAc_2$. The ability of the engineered *P. pastoris* strain to carry out the next step in human *N*-glycosylation was ascertained by screening a leader-GlcNAc transferase-I fusion library, similar to what was described for mannosidase. Several transformed strains were identified that produced $GlcNAcMan_5GlcNAc_2$–K3 by quantitatively converting $Man_5GlcNAc_2$ to the hybrid human intermediate.

Conclusion

Overall these results are highly significant in demonstrating that *N*-glycosylation pathways in *P. pastoris* can be extensively re-engineered without compromising the viability of host cells. This could not have been predicted because the cell itself must use *N*-glycosylation for its own cell-wall synthesis and there are no precedents that allow us to judge to what extent the formation of hybrid-type glycans would compromise the viability of the yeast. The requirement for proper intracellular retention and localization of the transformed glycosylation enzymes is clearly demonstrated by showing that only a few combinations of leader or catalytic domain fusions have a strong effect on *in vivo* mannosidase trimming. In addition, the substrate-limiting function of sugar nucleotide-transporters is demonstrated by showing that the absence of these transporters leads to inefficient GlcNAc transfer, presumably because of limiting amounts of UDP-GlcNAc in the Golgi of *P. pastoris*. Genetically modified *P. pastoris* strains have the potential to be further modifed to produce complex *N*-linked oligosaccharides and this, together with the demonstrated ability of *P. pastoris* to produce large quantities of expressed proteins, should be of great interest to the pharmaceutical industry and to other investigators.

Note added in proof

Just after this article was written, the synthesis of complex human glycoproteins in *P. pastoris* was reported in *Science*

(Hamilton, S.R., *et al.*, (2003) Production of complex human glycoproteins in yeast, *Science* 301, 1244–1246).

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Sugar coating extends half-lives and improves effectiveness of cytokine hormones

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Recombinant human erythropoietin (rhEPO) is an effective and widely used therapeutic agent that is produced by bioengineering. Modification of the rhEPO protein by glycoengineering increased its already abundant *N*-glycosylation, which enhances its erythropoietic activity *in vivo* by decreasing its metabolic clearance. Elliott *et al.* recently reported increased *in vivo* activities of thrombopoietin (Mpl ligand) and leptin following carbohydrate addition to both, which suggests that such glycoengineering could be applied to a variety of hormones, cytokines and growth factors.

Erythropoietin (EPO), thrombopoietin (Mpl ligand), and leptin are members of a family of structurally related cytokines, hormones and growth factors (Type 1 cytokines) that bind to specific, but structurally related surface receptors on their respective target cells [1]. The Type 1 cytokines include many interleukins and hematopoietic growth factors, such as EPO and Mpl ligand, as well as metabolic cytokines, such as growth hormone, prolactin and leptin. Each family member binds to the extracellular domains of a receptor dimer, thereby altering receptor conformation and initiating intracellular signalling. Although the specific sites of a Type 1 cytokine molecule that bind to its receptor are determined by the tertiary conformation of specific peptide sequences, the binding affinity can be affected by oligosaccharides that are covalently bound to asparagines (*N*-linked glycans), or serines or threonines (*O*-linked glycans) in areas of the molecule not directly involved in receptor binding. In addition to their effects on receptor binding, these oligosaccharides can affect the rate of metabolic clearance of the cytokine. Elliott *et al.* [2] recently demonstrated increased *in vivo* activities and durations of action by engineering hyperglycosylations of recombinant human (rh) EPO, Mpl ligand and leptin. The successful use of the hyperglycosylated rhEPO (darbepoetin alfa) in patients indicates that this glycoengineering technique, which resulted in prolonged action and increased activity of cytokine hormones, could be applied to other recombinant proteins that are used as therapeutic agents.

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Erythropoietin as a candidate for glycoengineering

EPO stimulates erythrocyte production by preventing apoptosis of erythroid progenitor cells during a specific period in their differentiation [3,4]. Intrinsic heterogeneity in the EPO-dependence of the erythroid progenitor cells results in increased erythrocyte production in response to

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